

60 REC d PCT/PTO 17 SEP 2001

FORM PTO-1390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES

213930US0PCT

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/926163

INTERNATIONAL APPLICATION NO

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/JP00/01608

16 March 2000

17 March 1999 (earliest)

TITLE OF INVENTION

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE THEREOF

APPLICANT(S) FOR DO/EO/US

Takashi SHIBATA, et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Notice of Consideration of Documents Cited in International Search Report

Notice of Priority, PCT/IB/304, PCT/IB/308

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) <b>09/1926163</b>		INTERNATIONAL APPLICATION NO. <b>PCT/JP00/01608</b>		ATTORNEY'S DOCKET NUMBER <b>213930US0PCT</b>	
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24. The following fees are submitted: <b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :</b> <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b> <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b> <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b> <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>	
				<b>\$860.00</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)). <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30				<b>\$130.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x \$18.00	<b>\$0.00</b>	
Independent claims	- 3 =	0	x \$80.00	<b>\$0.00</b>	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$990.00</b>	
<input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				<b>\$0.00</b>	
<b>SUBTOTAL =</b>				<b>\$990.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)). <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30				<b>\$130.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$1,120.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$1,120.00</b>	
				<b>Amount to be: refunded</b>	\$
				<b>charged</b>	\$

- a. ☒ A check in the amount of **\$1,120.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030**. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:



**22850**

**Surinder Sachar**  
Registration No. 34,423

SIGNATURE

**Norman F. Oblon**

NAME

**24,618**

REGISTRATION NUMBER

DATE

Sept. 17 2001

213930US0PCT



09/926,163, 127,101  
**Rec'd PCT/PTO 28 MAY 2002**

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :  
Takashi SHIBATA, et al. : GROUP ART UNIT:  
SERIAL NO: 09/926,163 :  
FILED: September 17, 2001 : EXAMINER:  
FOR: SORBITOL DEHYDROGENASE, GENE  
ENCODING THE SAME AND USE THEREOF

RESPONSE AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

Responsive to the Official Correspondence dated April 24, 2002, Applicants submit herewith a substitute computer-readable Sequence Listing. The paper-copy substitute Sequence Listing was timely filed on March 5, 2002, along with a corresponding computer-readable Sequence Listing.

REMARKS

Claims 1-48 are active in the present application. Favorable consideration is respectfully requested.

Applicants have now submitted a substitute computer-readable Sequence Listing. Contents of the paper copy of the Substitute Sequence Listing filed on March 5, 2002, and the substitute computer-readable Sequence Listing filed herewith are identical. Support for all

the sequences listed in the substitute computer-readable Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



Norman F. Oblon  
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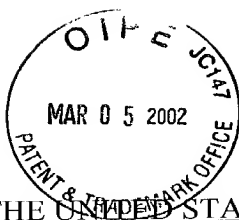


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213930US-0-PCT



IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :  
 Takashi SHIBATA, et al. :  
 SERIAL NO: 09/926,163 : ATTN: APPLICATION BRANCH  
 FILED: September 17, 2001 :  
 FOR: SORBITOL DEHYDROGENASE, GENE  
 ENCODING THE SAME AND USE THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
 WASHINGTON, D.C. 20231

SIR:

Applicants submit herewith a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as follows:

Page 66 (Abstract), after the last line, beginning on the next page, please deleted the Sequence Listing at pages insert the substitute Sequence Listing attached hereto.

REMARKS

Claims 1-48 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence

Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



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09/926163  
PTO/PCT Rec'd 21 DEC 2001

213930US-0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :  
TAKASHI SHIBATA ET AL. :  
SERIAL NO: 09/926,163 : ATTN: APPLICATION BRANCH  
FILED: SEPTEMBER 17, 2001 :  
FOR: SORBITOL DEHYDROGENASE,  
GENE ENCODING THE SAME AND  
USE THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as shown in the attached marked-up copy to read as follows:

Page 4, beginning at line 22 and continuing to page 5, line 2, delete the paragraph and replace it with the following paragraph:

(5) An SLDH which is the following protein (a) or (b):

(a) a protein consisting of an amino acid sequence depicted in Sequence Listing SEQ

ID NO:2

(b) a protein consisting of the same amino acid sequence as

(a) above, except that one to several amino acids are deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

Page 5, lines 5-10, delete the paragraph and replace it with the following paragraph:

(7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

Page 5, lines 13-16, delete the paragraph and replace it with the following paragraph:

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1 and a partial DNA thereof.

Page 5, beginning at line 20 and continuing to page 6, line 1, delete the paragraph and replace it with the following paragraph:

(11) A promoter gene comprising the DNA of the following (a) or (b)

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA having a base sequence of the above-mentioned ~(a) wherein one to several bases is (are) deleted, substituted, inserted, added or modified, which DNA shows a promoter activity at least in one microorganism.

Page 8, beginning at line 21 and continuing to page 9, line 21, delete the paragraph and replace it with the following paragraph:

The SLDH of the present invention is not particularly limited as regards the derivation as long as it shows the above-mentioned characteristics. It may be derived from a naturally occurring organism, a spontaneous or artificial mutant, or a transformant which is obtained by introducing a heterologous (i.e. foreign) SLDH gene. Preferably, SLDH derived

from acetic acid bacteria, particularly bacteria belonging to the genus *Gluconobacter*, more preferably *Gluconobacter oxydans*, particularly the strain *Gluconobacter oxydans* 6624 (FERM BP-4415; International Patent Publication No. W095/23220) are exemplified. In another preferable mode, the SLDH of the present invention is an SLDH derived from the same gene as is the SLDH derived from the strain *G. oxydans* 6624 in its molecular evolution. As used herein, by the "derived from the same gene ... in its molecular evolution" is meant an SLDH reasonably concluded to have evolved from the same gene as has an SLDH derived from strain *G. oxydans* 6624 in its molecular evolution, as a result of the analyses of DNA sequence, physiological role and the like, and their DNA sequences show high homology. These SLDHs preferably have not less than 60%, most preferably not less than 80%, homology in the DNA sequence with an SLDH derived from the strain *G. oxydans* 6624. These genes can be cloned based on the DNA sequence depicted in Sequence Listing SEQ ID NO:1 and using a suitable primer according to the PCR method or using a suitable probe according to the hybridization method, as detailed later.

Page 9, beginning at line 22 and continuing to page 10, line 8, delete the paragraph and replace it with the following paragraph:

In a more preferable mode, the SLDH of the present invention is a protein having an amino acid sequence depicted in Sequence Listing SEQ ID NO:2 or a protein having an amino acid sequence having the amino acid sequence comprising one to several amino acids deleted, substituted, inserted, added or modified, as long as the SLDH activity is not impaired.

Page 11, lines 11-16, delete the paragraph and replace it with the following paragraph:

Production of the SLDH of the present invention by chemical synthesis includes the steps of, for example, synthesizing, based on the amino acid sequence depicted in Sequence Listing SEQ ID NO:2, the entirety or a part of each sequence using peptide synthesizer, and renaturing the obtained polypeptide under suitable renaturation conditions.

Page 17, lines 9-26, delete the paragraph and replace it with the following paragraph:

A DNA encoding the SLDH of the present invention preferably encodes an amino acid sequence depicted in v. Sequence Listing SEQ ID NO:2, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted, substituted, inserted or added (provided that a protein consisting of the mutated amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

Page 18, lines 6-14, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO: 1.

Page 18, lines 15-19, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:1.

Page 18, beginning at line 20 and continuing to page 19, line 8, delete the paragraph and replace it with the following paragraph:

The DNA encoding SLDH, which is obtained from a genomic DNA with the SLDH activity as an index as mentioned above, contains a promoter gene sequence in the 5' upstream region. This promoter gene preferably has a base sequence having a base number 1 - 536 from the base sequence depicted in Sequence Listing SEQ ID NO:1, or said base sequence wherein one to several amino acids are deleted, substituted, inserted, added or modified, which is a DNA having a promoter activity in at least one microorganism. As the "microorganism" here, there are preferably exemplified prokaryotes such as bacteria (e.g., *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas*, *Gluconobacter*, *Pseudogluconobacter*, *Acetobacter* and the like) and actinomyces, and certain eucaryotes such as yeast and the like.

Page 35, lines 16-26, delete the paragraph and replace it with the following paragraph:

Using plasmids pUCP19-HC, pUC18-S1, pUC18-ES and pUC18E1 as templates and using universal primer and reverse primer (New England Labs.), which were M13 sequencing primers, first sequencing was performed. The sample was fluorescent labeled with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The following 11 kinds of primers were synthesized and using pUCP19-HC as a template sequencing was performed, whereby the

base sequence of about 4 kb Hind IIIHind III fragment was determined (Sequence Listing SEQ ID NO:1).

Page 48, after the last line beginning on a new page, please replace the original Sequence Listing with the substitute Sequence Listing attached hereto.

# IN THE CLAIMS

Please amend the claims as shown on the marked-up copy following this amendment to read as follows.

6. (Amended) A DNA encoding the sorbitol dehydrogenase as claimed in claim 1.
8. (Amended) The DNA of claim 6, which is derived from bacteria belonging to the genus *Gluconobacter*.
12. (Amended) A recombinant vector comprising a DNA as claimed in claim 6.
13. (Amended) An expression vector comprising a DNA as claimed in claim 6.
15. (Amended) A transformant obtained by transforming a host cell with an expression vector of claim 13.
17. (Amended) The transformant of claim 15, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.
18. (Amended) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium and harvesting the sorbitol dehydrogenase of claim 1 or the protein of claim 10 from the obtained culture.
22. (Amended) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-



gluconic acid obtained by the method of claim 20 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

Please add new Claims 23-48.

23. (New) A DNA encoding the sorbitol dehydrogenase as claimed in claim 5.

24. (New) The DNA of claim 23, which is (a) or (b) in the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

25. (New) The DNA of claim 23, which is derived from bacteria belonging to the genus *Gluconobacter*.

26. (New) A recombinant vector comprising a DNA as claimed in claim 23.

27. (New) A recombinant vector comprising a DNA as claimed in claim 9.

28. (New) An expression vector comprising a DNA as claimed in claim 23.

29. (New) An expression vector comprising a DNA as claimed in claim 9.

30. (New) The expression vector of claim 28 further comprising a DNA encoding sorbose dehydrogenase and/or a DNA encoding sorbosone dehydrogenase.

31. (New) The expression vector of claim 29 further comprising a DNA encoding sorbose dehydrogenase and/or a DNA encoding sorbosone dehydrogenase.

32. (New) A transformant obtained by transforming a host cell with an expression vector of claim 28.

33. (New) A transformant obtained by transforming a host cell with an expression vector of claim 29.

34. (New) The transformant of claim 32, which belongs to a genus selected from the group consisting of *Escherichia coli*, the genus *Pseudomonas*, the genus *Gluconobacter*, the genus *Acetobacter* and the genus *Pseudogluconobacter*.

35. (New) The transformant of claim 33, which belongs to a genus selected from the group consisting of *Escherichia coli*, the genus *Pseudomonas*, the genus *Gluconobacter*, the genus *Acetobacter* and the genus *Pseudogluconobacter*.

36. (New) The transformant of claim 32, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.

37. (New) The transformant of claim 33, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.

38. (New) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium harvesting the sorbitol dehydrogenase having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)<sup>+</sup> dependent

(d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or

a protein derived from the genus *Gluconobacter*, which is encoded by a gene encoding a protein having a sorbitol dehydrogenase activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1 and a partial DNA thereof, and which has sorbitol dehydrogenase activity, from the obtained culture.

- (d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or a protein derived from the genus *Gluconobacter*, which is encoded by a gene encoding a protein having a sorbitol dehydrogenase activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof, and which has sorbitol dehydrogenase activity, from the obtained culture.

40. (New) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 29 in a medium and harvesting the sorbitol dehydrogenase having the following properties

- (d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or

a protein derived from the genus *Gluconobacter*, which is encoded by a gene encoding a protein having a sorbitol dehydrogenase activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1 and a partial DNA thereof, and which has sorbitol dehydrogenase activity, from the obtained culture.

41. (New) A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an expression vector of claim 28 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

42. (New) A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an expression vector of claim 29 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

43. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing a host cell transformed with an expression vector containing a DNA encoding sorbose dehydrogenase and a DNA encoding corbosone dehydrogenase in a medium and bringing the L-sorbose obtained according to the method of claim 41 into contact with the obtained culture or a treated product thereof.

44. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing a host cell transformed with an expression vector containing a DNA encoding sorbose dehydrogenase and a DNA encoding corbosone dehydrogenase in a medium and bringing the L-sorbose obtained according to the method of claim 42 into contact with the obtained culture or a treated product thereof.

45. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing the transformant of claim 36 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

46. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing the transformant of claim 32 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

47. (New) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gluconic acid obtained by the method of claim 43 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

48. (New) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gluconic acid obtained by the method of claim 44 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

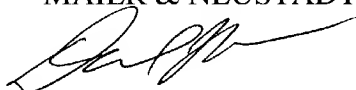
REMARKS

Claims 1-48 are active in the present application. Claims 6, 8, 12-13, 15, 17-18 and 22 have been amended to remove multiple dependencies. Claims 23-48 are new claims. Support for the new claims is found in the original claims. The specification is amended to include a substitute Sequence Listing and to change the Sequence Identifiers (SEQ ID NO:) to correspond to the substitute Sequence Listing.

Applicants submit that the sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the substitute Sequence Listing. Support for all of the sequences listed in the substitute Sequence Listing is found in the present application as originally filed. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the foregoing amendments. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



Norman F. Oblon  
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Registration No. 24,618

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**Marked-Up Copy**

Serial No:

09/926,163

Amendment Filed on:

12-21-01IN THE SPECIFICATION

Please amend the specification as shown in the attached marked-up copy to read as follows:

Page 4, beginning at line 22 and continuing to page 5, line 2, delete the paragraph and replace it with the following paragraph:

(5) An SLDH which is the following protein (a) or (b):

(a) a protein consisting of an amino acid sequence depicted in Sequence Listing SEQ

ID NO:[1]2

(b) a protein consisting of the same amino acid sequence as

(a) above, except that one to several amino acids are deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

Page 5, lines 5-10, delete the paragraph and replace it with the following paragraph:

(7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

Page 5, lines 13-16, delete the paragraph and replace it with the following paragraph:

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1 and a partial DNA thereof.

Page 5, beginning at line 20 and continuing to page 6, line 1, delete the paragraph and replace it with the following paragraph:

(11) A promoter gene comprising the DNA of the following (a) or (b)

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1

(b) a DNA having a base sequence of the above-mentioned ~(a) wherein one to several bases is (are) deleted, substituted, inserted, added or modified, which DNA shows a promoter activity at least in one microorganism.

Page 8, beginning at line 21 and continuing to page 9, line 21, delete the paragraph and replace it with the following paragraph:

The SLDH of the present invention is not particularly limited as regards the derivation as long as it shows the above-mentioned characteristics. It may be derived from a naturally occurring organism, a spontaneous or artificial mutant, or a transformant which is obtained by introducing a heterologous (i.e. foreign) SLDH gene. Preferably, SLDH derived from acetic acid bacteria, particularly bacteria belonging to the genus *Gluconobacter*, more preferably *Gluconobacter oxydans*, particularly the strain *Gluconobacter oxydans* 6624 (FERM BP-4415; International Patent Publication No. W095/23220) are exemplified. In another preferable mode, the SLDH of the present invention is an SLDH derived from the same gene as is the SLDH derived from the strain *G. oxydans* 6624 in its molecular evolution. As used herein, by the "derived from the same gene ... in its molecular evolution" is meant an SLDH reasonably concluded to have evolved from the same gene as has an



SLDH derived from strain *G. oxydans* 6624 in its molecular evolution, as a result of the analyses of DNA sequence, physiological role and the like, and their DNA sequences show high homology. These SLDHs preferably have not less than 60%, most preferably not less than 80%, homology in the DNA sequence with an SLDH derived from the strain *G. oxydans* 6624. These genes can be cloned based on the DNA sequence depicted in Sequence Listing SEQ ID NO:[2]1 and using a suitable primer according to the PCR method or using a suitable probe according to the hybridization method, as detailed later.

Page 9, beginning at line 22 and continuing to page 10, line 8, delete the paragraph and replace it with the following paragraph:

In a more preferable mode, the SLDH of the present invention is a protein having an amino acid sequence depicted in Sequence Listing SEQ ID NO:[1]2 or a protein having an amino acid sequence having the amino acid sequence comprising one to several amino acids deleted, substituted, inserted, added or modified, as long as the SLDH activity is not impaired.

Page 11, lines 11-16, delete the paragraph and replace it with the following paragraph:

Production of the SLDH of the present invention by chemical synthesis includes the steps of, for example, synthesizing, based on the amino acid sequence depicted in Sequence Listing SEQ ID NO:[1]2, the entirety or a part of each sequence using peptide synthesizer, and renaturing the obtained polypeptide under suitable renaturation conditions.

Page 17, lines 9-26, delete the paragraph and replace it with the following paragraph:

A DNA encoding the SLDH of the present invention preferably encodes an amino acid sequence depicted in v. Sequence Listing SEQ ID NO:[1]2, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted,

substituted, inserted or added (provided that a protein consisting of the mutated amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

Page 18, lines 6-14, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:[2] 1.

Page 18, lines 15-19, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO[2]1.

Page 18, beginning at line 20 and continuing to page 19, line 8, delete the paragraph and replace it with the following paragraph:

Using plasmids pUCP19-HC, pUC18-S1, pUC18-ES and pUC18E1 as templates and using universal primer and reverse primer (New England Labs.), which were M13 sequencing primers, first sequencing was performed. The sample was fluorescent labeled with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The following 11 kinds of primers were synthesized and using pUCP19-HC as a template sequencing was performed, whereby the base sequence of about 4 kb Hind IIIHind III fragment was determined (Sequence Listing SEQ ID NO:[2]1).

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# IN THE CLAIMS

Please amend the claims as follows.

-- 6. (Amended) A DNA encoding the sorbitol dehydrogenase [of any of claims 1 to 5] as claimed in claim 1.

8. (Amended) The DNA of claim 6 [or 7], which is derived from bacteria belonging to the genus *Gluconobacter*.

12. (Amended) A recombinant vector comprising a DNA [of any of claims 6 to 9] as claimed in claim 6.

13. (Amended) An expression vector comprising a DNA [of any of claims 6 to 9] as claimed in claim 6.

15. (Amended) A transformant obtained by transforming a host cell with an expression vector of claim 13 [or 14].

17. (Amended) The transformant of claim 15 [or 16], which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.

18. (Amended) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium and harvesting the sorbitol dehydrogenase [of any of claims 1 to 5] having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)<sup>+</sup> dependent

(d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or [the protein of claim 10] a protein derived



09/926163  
PTO/PCT Rec'd 21 DEC 2001

SEQUENCE LISTING

<110> SHIBATA, Takashi  
ICHIKAWA, Chiyo  
MATSUURA, Mitsutaka  
NOGUCHI, Yuji  
SAITO, Yoshimasa  
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TAKATA, Yoko

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16

PTO/PCT Rec'd 21 DEC 2001

## SPECIFICATION

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE  
THEREOF

## TECHNICAL FIELD

5 This invention relates to a novel sorbitol dehydrogenase (in the present invention, sorbitol dehydrogenase means an enzyme capable of catalyzing a reaction for converting D-sorbitol to L-sorbose by oxidation; hereinafter to be referred to as SLDH), a gene encoding the  
10 same, a method for producing L-sorbose and 2-keto-L-gulonic acid (hereinafter to be referred to as 2KLGA) by gene manipulation using said gene, and an expression system involved in the production thereof. The present invention further relates to a method for producing L-ascorbic acid or  
15 a salt thereof utilizing the 2KLGA obtained by the above-mentioned method.

## BACKGROUND ART

L-sorbose is an important intermediate for the synthesis of L-ascorbic acid (vitamin C) by the Reichstein  
20 method (see Fig. 1). When D-sorbitol is chemically oxidized, approximately a half of the product becomes D-sorbose, whereas when D-sorbitol is brought into contact with a microorganism having an SLDH activity, only an L-enantiomer is obtained in a yield of about 95%. Therefore, a  
25 fermentation method has been conventionally used for converting D-sorbitol to L-sorbose.

On the other hand, 2KLGA is industrially synthesized by chemically oxidizing L-sorbose. There are known microorganisms that convert L-sorbose into 2KLGA by a two-step enzymatic oxidation reaction by L-sorbose dehydrogenase (SDH) and L-sorbose dehydrogenase (SNDH), but the production amount of 2KLGA is low by these methods.

As a method by which to produce 2KLGA more efficiently than before by a fermentation method, there is mentioned a method comprising isolating an SLDH gene, introducing the gene into a microorganism having an SDH or SNDH activity to give a recombinant microorganism capable of synthesizing 2KLGA from D-sorbitol, and bringing the microorganism into contact with D-sorbitol.

Several types of SLDHs have been isolated [Agric. Biol. Chem., 46(1), 135-141 (1982); Biokhimiia, 43(6), 1067-1078 (1978); J. Biol. Chem., 224, 323 (1957); J. Biol. Chem., 226, 301 (1957); J. Bacteriol., 71, 737 (1956)]. The present inventors have already isolated, from a strain belonging to *Gluconobacter oxydans*, a gene encoding SLDH which is of a membrane-bound type, consists of two large and small subunits and which binds with cytochrome c-like polypeptide and acts (international patent publication No. WO99/20763). However, there is no report on the cloning of a different type of SLDH gene.

It is therefore an object of the present invention to provide a novel SLDH gene useful for the fermentative

production of 2KLGA, and to provide a host microorganism transformed with said gene, particularly a transformant obtained by introducing said gene into a host already having SDH and SNDH activity, or a transformant obtained by

5 introducing said gene together with SDH gene and SNDH gene.

Another object of the present invention is to provide a method for producing L-sorbose or 2KLGA from D-sorbitol using said microorganism, and to provide a method for producing L-ascorbic acid from 2KLGA obtained by this method. It is yet

10 another object of the present invention to provide a method for producing a recombinant SLDH by culture of a host microorganism transformed with said SLDH gene and a method for producing L-sorbose by an enzyme method using said SLDH.

#### DISCLOSURE OF THE INVENTION

15 The present inventors have conducted intensive studies in an attempt to solve the above-mentioned problems and succeeded in cloning a DNA containing a coding region of SLDH from a chromosomal DNA library of a strain belonging to the genus *Gluconobacter* having said enzyme activity. As a result

20 of the sequencing, the DNA was confirmed to contain a novel SLDH gene completely different from the SLDH gene previously isolated by the present inventors. Moreover, the present inventors transformed *Pseudomonas* with an expression vector containing the DNA and succeeded in purifying a recombinant

25 SLDH from the culture of said recombinant *Pseudomonas*. They have also transformed *Pseudomonas* transformed with an

expression vector containing said DNA, with an expression  
vector containing an SDH gene and an SNDH gene and  
efficiently converting D-sorbitol to 2KLGA using the culture  
of this transformant, which resulted in the completion of the  
5 present invention.

Accordingly, the present invention provides the  
following.

- (1) An SLDH having the following physicochemical properties:
  - (a) action: catalyzes the reaction converting D-sorbitol to  
10 L-sorbose
  - (b) molecular weight: about 54 kDa
  - (c) coenzyme: NAD(P)<sup>+</sup> dependent
  - (d) substrate specificity: specifically oxidizes sorbitol,  
mannitol and arabitol, but does not act on xylitol, ribitol,  
15 inositol and glycerol.
- (2) The SLDH of the above-mentioned (1), which is derived  
from the strain *Gluconobacter oxydans* G624.
- (3) An SLDH which is originated from the same gene as is the  
SLDH of the above-mentioned (2) in its molecular evolution.
- 20 (4) The SLDH of the above-mentioned (3), which is derived  
from a bacteria belonging to the genus *Gluconobacter*.
- (5) An SLDH which is the following protein (a) or (b):
  - (a) a protein consisting of an amino acid sequence depicted  
in Sequence Listing SEQ ID NO:1
  - 25 (b) a protein consisting of the same amino acid sequence as  
(a) above, except that one to several amino acids are



deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

(6) A DNA encoding the SLDH of any of the above-mentioned (1) to (5).

5 (7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2

(b) a DNA capable of hybridizing to the base sequence of the  
10 above-mentioned (a) under stringent conditions.

(8) The DNA of the above-mentioned (6) or (7), which is derived from bacteria belonging to the genus *Gluconobacter*.

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence  
15 of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof.

(10) A protein derived from the genus *Gluconobacter*, which is encoded by the gene of the above-mentioned (9) and which has an SLDH activity.

20 (11) A promoter gene comprising the DNA of the following (a) or (b):

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:2

(b) a DNA having a base sequence of the above-mentioned (a)  
25 wherein one to several bases is(are) deleted, substituted, inserted, added or modified, which DNA shows a promoter

activity at least in one microorganism.

(12) A recombinant vector comprising a DNA of any of the above-mentioned (6) to (9).

(13) An expression vector comprising a DNA of any of the  
5 above-mentioned (6) to (9).

(14) The expression vector of the above-mentioned (13), further comprising a DNA encoding an SDH and/or a DNA encoding an SNDH.

(15) A transformant obtained by transforming a host cell  
10 with an expression vector of the above-mentioned (13) or (14).

(16) The transformant of the above-mentioned (15), which belongs to a genus selected from the group consisting of *Escherichia coli*, the genus *Pseudomonas*, the genus  
15 *Gluconobacter*, the genus *Acetobacter* and the genus *Pseudogluconobacter*.

(17) The transformant of the above-mentioned (15) or (16), which is capable of converting D-sorbitol to 2-KLGA.

(18) A method for producing a protein having an SLDH  
20 activity, which method comprises culturing a host cell transformed with an expression vector of the above-mentioned (13) in a medium and harvesting the SLDH of any of the above-mentioned (1) to (5) or the protein of (10) from the obtained culture.

25 (19) A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an





derived from acetic acid bacteria, particularly bacteria  
belonging to the genus *Gluconobacter*, more preferably  
*Gluconobacter oxydans*, particularly the strain *Gluconobacter*  
*oxydans* G624 (FERM BP-4415; International Patent Publication  
5 No. WO95/23220) are exemplified. In another preferable mode,  
the SLDH of the present invention is an SLDH derived from the  
same gene as is the SLDH derived from the strain *G. oxydans*  
G624 in its molecular evolution. As used herein, by the  
"derived from the same gene ... in its molecular evolution" is  
10 meant an SLDH reasonably concluded to have evolved from the  
same gene as has an SLDH derived from strain *G. oxydans* G624  
in its molecular evolution, as a result of the analyses of  
DNA sequence, physiological role and the like, and their DNA  
sequences show high homology. These SLDHs preferably have  
15 not less than 60%, most preferably not less than 80%,  
homology in the DNA sequence with an SLDH derived from the  
strain *G. oxydans* G624. These genes can be cloned based on  
the DNA sequence depicted in Sequence Listing SEQ ID NO:2 and  
using a suitable primer according to the PCR method or using  
20 a suitable probe according to the hybridization method, as  
detailed later.

In a more preferable mode, the SLDH of the present  
invention is a protein having an amino acid sequence depicted  
in Sequence Listing SEQ ID NO:1, or a protein having an amino  
25 acid sequence having the amino acid sequence comprising one  
to several amino acids deleted, substituted, inserted, added

or modified, as long as the SLDH activity is not impaired.

The SLDH of the present invention can be obtained by appropriately using (1) a method comprising isolating and purifying it from a culture of a cell or tissue as a starting  
5 material that produces the enzyme, (2) a method comprising chemical synthesis, (3) a method comprising purifying it from a cell manipulated by gene recombinant technique to express SLDH and the like.

The isolation and purification of SLDH from a naturally  
10 occurring SLDH producing cell includes, for example, the following steps. The cell is cultured in a suitable liquid medium, and a fraction having an SLDH activity is separated and recovered from the obtained culture. For example, when the enzyme is localized in cytosol (the SLDH of the present  
15 invention being  $\text{NAD(P)}^+$  dependent, localization in cytosol is expected), the culture is centrifuged and/or filtrated to recover the cell, and the cell is ruptured by ultrasonication, lysozyme treatment, osmotic pressure shock and the like and centrifuged at about 10,000 - 40,000 rpm to  
20 recover a supernatant (soluble fraction). The objective SLDH can be purified from the obtained soluble fraction by appropriately combining separation techniques conventionally used for separation and purification of enzyme proteins. Such separation techniques include, for example, methods  
25 utilizing difference in solubility such as salting out, solvent precipitation method and the like, methods utilizing

Production of the SLDH of the present invention by chemical synthesis includes the steps of, for example, synthesizing, based on the amino acid sequence depicted in Sequence Listing SEQ ID NO:1, the entirety or a part of each  
15 sequence using peptide synthesizer, and renaturing the obtained polypeptide under suitable renaturation conditions.

following, cloning a DNA encoding the enzyme from the DNA of a cell having this enzyme, and adding, to this DNA by gene manipulation, a nucleotide sequence encoding an amino acid sequence capable of adsorbing to a metal ion chelate.

5 An enzyme gene can be generally cloned by the following method. A desired enzyme is purified completely or partially from a cell or tissue that produces the enzyme by the above-mentioned method, and the N terminal amino acid sequence is determined by the Edman method. The enzyme is partially  
10 digested by a sequence-specific protease and the amino acid sequence of the obtained oligopeptide is also determined by the Edman method. Oligonucleotides having base sequences corresponding to the amino acid sequences thus determined are synthesized, and using these as primers or probes, a DNA  
15 encoding this enzyme is cloned from RNA or DNA prepared from a cell or tissue capable of producing the enzyme, by the PCR method or colony (or plaque) hybridization method.

Alternatively, an antibody against the enzyme is prepared using the entirety or a part of a completely or  
20 partially purified enzyme as an antigen by a conventional method, and a DNA encoding the enzyme can be cloned from a cDNA or genomic DNA library prepared from a cell or tissue capable of producing the enzyme, by immunoscreening.

However, in the case of an enzyme that is unstable and  
25 whose purification is difficult, such as the above-mentioned SLDH derived from *G. oxydans* G624, the gene of the enzyme can



be screened using its enzyme activity as a marker, as a fragment containing its promoter sequence from the genomic DNA library. Inasmuch as SLDH converts D-sorbitol to L-sorbose, a clone having an SLDH activity can be selected by  
5 detecting the generated L-sorbose. Note that the application of this method often accompanies technical difficulty.

To be specific, a chromosomal DNA is isolated from a cell or tissue having an SLDH activity by a conventional method, and digested by a suitable restriction enzyme, preferably partially digested by a restriction enzyme having many restriction sites in a chromosomal DNA, and the obtained fragment is inserted in a suitable cloning vector. As the cloning vector, exemplified are plasmid vector, phage vector and the like. Because of the capability of accommodating a large DNA insert and recovering as a colony, a cosmid vector and a charomid vector are preferable. When a phage vector, a cosmid vector and the like are used, *in vitro* packaging is further applied to obtain a genomic DNA library.

When a cosmid library is used, a suitable indicating  
20 bacteria, preferably *Escherichia coli* competent cell having  
high transformation capability, after infection with a  
packaging solution obtained as above, is plated on a solid  
medium and cultured. The resulting respective colonies are  
individually inoculated to a liquid medium containing D-  
25 sorbitol and cultured. After the completion of the culture,  
a culture supernatant is recovered and candidate clones

having an SLDH activity are selected using, for example, a color identification reaction with ketohexose, such as a resorcin-hydrochloric acid reaction (Cohen, *J. Biol. Chem.*, 201, 71, 1953), a resorcin-ferric salt-hydrochloric acid reaction (Kulka, *Biochem. J.*, 63, 542, 1956) and the like.

The presence of SLDH activity (conversion of D-sorbitol to L-sorbose) in the obtained clone is confirmed by the detection of sorbose in the culture supernatant by, for example, HPLC and the like.

Because the DNA insert of cosmid clone is considerably large (35 - 45 kb), a part of a non-SLDH gene region of the insert DNA is desirably removed for downsizing for facilitated subcloning to plasmid. For downsizing of the DNA insert, for example, subcloning to a charomid vector and the like is employed. Since a charomid vector has a spacer DNA of various lengths, DNA having various lengths smaller than a cosmid vector can be cloned. In the present invention, for example, a charomid vector capable of accommodating an about 10 - 20kb DNA insert is preferably used. The charomid clone having an SLDH activity can be selected according to the method mentioned above.

The subcloning to a plasmid vector can be done by, for example, applying plural charomid clones obtained as mentioned above to restriction enzyme mapping, downsizing a DNA insert using a restriction enzyme found to have no restriction site in the SLDH gene, and ligating with a

plasmid vector that underwent a restriction enzyme treatment.

Apart from the above-mentioned strategy, moreover, a DNA encoding the SLDH of the present invention can be directly cloned using the PCR method. That is, PCR is conducted according to a conventional method, using a genomic DNA or cDNA (or mRNA) derived from a cell or tissue having the enzyme activity as a template, and using a pair of oligonucleotides, where an amplification fragment suitably covers the coding region of SLDH, as a primer to amplify a DNA fragment containing the coding region of SLDH. This method is particularly useful for cloning of an SLDH gene having the same origin in the molecular evolution with an SLDH having a known sequence. For example, when an SLDH gene derived from a bacteria, which is speculated to have the same origin in the molecular evolution with an SLDH derived from the strain *G. oxydans* G624, is to be cloned, sense and antisense primers capable of amplifying a DNA fragment having high homology with a DNA fragment containing a base sequence of base number 537 - 1991 from the sequence is constructed based on the DNA sequence depicted in Sequence Listing SEQ ID NO:2 and the PCR method is performed. When the DNA sequence of SLDH having high homology with the objective SLDH is unknown, for example, PCR is performed using some sequences conserved relatively well in the 5' upstream region as sense primers, and some complementary strand sequences conserved relatively well in the 3' downstream region as antisense

primers to clone the SLDH gene. When the upstream and downstream sequences of SLDH are unknown, the annealing temperature needs to be set lower, so that a template DNA and a primer to be used containing some mismatches can still be bound. Therefore, the PCR product may be a mixture of a fragment containing the objective SLDH gene and a non-specific amplification fragment. In this case, the obtained amplification fragment is cloned to a suitable cloning vector (for example, plasmid vector for TA cloning and the like).

When the objective amplification fragment does not contain a promoter region, the obtained amplification fragment is cloned to an expression vector, with which a competent cell, such as *Escherichia coli*, is transformed, and the transformant having an SLDH activity is screened by the aforementioned method.

As a different strategy for the cloning of an SLDH gene having the same origin in the molecular evolution with an SLDH having a known sequence, direct cloning by hybridization method such as Southern method and the like may be employed, wherein a genomic DNA or cDNA (or mRNA) derived from a cell or tissue having an SLDH activity is used as a template and the entirety or a part of a known DNA sequence is used as a probe. The conditions of the hybridization may be an appropriately altered stringency depending on the origin of the DNA. For example, the conditions may be changed based on the degree of closeness in the relation of the microorganism

A DNA encoding the SLDH of the present invention preferably encodes an amino acid sequence depicted in Sequence Listing SEQ ID NO:1, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted, substituted, inserted or added (provided that a protein consisting of the mutated amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

The "stringent conditions" here mean those under which a DNA having about not less than 60% homology of base sequence can hybridize. The stringency can be controlled by appropriately changing the temperature, salt concentration and the like of hybridization reaction and washing.

Another DNA of the present invention also encompasses a gene consisting of a base sequence capable of hybridizing to a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof, and encoding a protein having an SLDH activity. Therefore, a protein having an SLDH activity, which is encoded by this gene, particularly a protein derived from the genus *Gluconobacter*, is also within the scope of the present invention.

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:2.

The DNA encoding SLDH, which is obtained from a genomic DNA with the SLDH activity as an index as mentioned above, contains a promoter gene sequence in the 5' upstream region. This promoter gene preferably has a base sequence having a base number 1 - 536 from the base sequence depicted in Sequence Listing SEQ ID NO:2, or said base sequence wherein one to several amino acids are deleted, substituted,



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expression of SLDH in which the specific amino acid sequence has been added to the terminus of the original SLDH amino acid sequence. The specific amino acid sequence capable of accelerating the purification of SLDH is exemplified by amino acid sequence capable of adsorbing to a metal ion chelate, preferably a sequence consisting of basic amino acids such as histidine, lysine, arginine and the like, more preferably a sequence consisting of histidine. Such sequence can be added to the terminus of amino or carboxyl of SLDH, with preference given to addition to the carboxyl terminus. Such modified SLDH coding sequence can be constructed by synthesizing an oligonucleotide wherein a base sequence encoding the amino acid sequence to be added is added to a base sequence consistent with a terminus sequence of the inherent SLDH coding sequence, and, using this as one of the primers and SLDH DNA as a template, performing PCR. The resulting recombinant SLDH can be quickly isolated and purified using a carrier on which a metal ion chelate capable of adsorbing the added amino acid sequence has been immobilized, as detailed in the following.

When an expression vector containing a DNA encoding the SLDH of the present invention is used for the production of 2KLGA, an expression vector containing, in addition to the DNA, a DNA encoding SDH and/or SNDH in a form permitting expression in the host cell may be used. The DNA encoding SLDH, a DNA encoding SDH and a DNA encoding SNDH may be

placed under control of different promoters, or two of which or more may be placed in tandem under the control of the same promoter.

The transformant of the present invention can be prepared by transforming a host cell with a recombinant vector containing a DNA encoding the SLDH of the present invention. The host cell is not particularly limited as long as it can be adapted to the recombinant vector to be used and can be transformed, and various cells conventionally used in this field, such as a naturally occurring cell or an artificially produced mutant cell or a recombinant cell, can be utilized. Preferably, bacteria, particularly *Escherichia coli* (e.g., DH5 , HB101 and the like), *Bacillus subtilis*, the genus *Pseudomonas* bacteria (e.g., *Pseudomonas fluorescence* and the like), the genus *Gluconobacter* bacteria (e.g., *Gluconobacter oxydans* and the like), the genus *Pseudogluconobacter* bacteria, the genus *Acetobacter* bacteria and the like are used.

A recombinant vector can be introduced into a host cell by a method conventionally known. For example, when the host is bacteria such as *Escherichia coli*, *Bacillus subtilis* and the like, the method of Cohen et al. [*Proc. Natl. Acad. Sci. USA*, 69: 2110 (1972)], protoplast method [*Mol. Gen. Genet.*, 168: 111 (1979)], competent method [*J. Mol. Biol.*, 56: 209 (1971)], electroporation method and the like can be used.

Particularly, the transformant of the present invention

is a host cell transformed with an expression vector containing a DNA encoding the SLDH of the present invention. When the transformant is prepared with the aim of producing 2KLGA from D-sorbitol, the host cell needs to have an ability to convert L-sorbose to 2KLGA. Preferably, the host cell produces SDH and SNDH activity. Such naturally occurring cell is, for example, bacteria belonging to the genus *Gluconobacter*, the genus *Acetobacter*, the genus *Pseudogluconobacter* and the like, specifically *Gluconobacter oxydans* T-100 (FERM BP-4415; International Patent Publication No. WO95/23220) and the like. Such artificially prepared cell is, for example, a cell transformed with an expression vector functionally containing a DNA encoding SDH and SNDH isolated from the above-mentioned naturally occurring bacteria and the like, preferably *Escherichia coli*, the genus *Pseudomonas* bacteria, the genus *Gluconobacter* bacteria, the genus *Pseudogluconobacter* bacteria, the genus *Acetobacter* bacteria and the like. Specifically, *E. coli* JM109-pUC19SD5 (International Patent Publication No. WO94/20609), *Gluconobacter oxydans* NB6939-pSDH-tufB1, *Gluconobacter oxydans* NB6939-pSDH-trp6, *Gluconobacter oxydans* NB6939-pSDH-PL1, *Gluconobacter oxydans* NB6939-pSDH-tac8 (all from International Patent Publication No. WO95/23220) and the like are exemplified.

The transformant of the present invention can be also obtained by transforming a host cell with an expression

vector containing, in addition to the above-mentioned DNA encoding SLDH, a DNA encoding SDH and/or a DNA encoding SNDH in a form permitting expression in the host cell. When the expression vector lacks one of the DNA encoding SDH and the DNA encoding SNDH, the host cell may be co-transformed along with a different expression vector containing said DNA.

The recombinant SLDH of the present invention can be produced by culturing a transformant containing an expression vector containing a DNA encoding the above-mentioned SLDH in a suitable medium and harvesting SLDH from the obtained culture.

The nutrient medium to be used contains, as a carbon source, saccharides such as glucose and fructose, glycerol, preferably L-sorbose and D-sorbitol. It may contain an inorganic or organic nitrogen source (e.g., ammonium sulfate, ammonium chloride, hydrolysate of casein, yeast extract, polypeptone, bactotrypton, beef extract and the like). When desired, other nutrient sources [e.g., inorganic salt (e.g., sodium diphosphate or potassium diphosphate, potassium hydrogenphosphate, magnesium chloride, magnesium sulfate, calcium chloride), vitamins (e.g., vitamin B1), antibiotics (e.g., ampicillin, kanamycin) etc.] may be added to the medium. Preferably, the medium contains D-sorbitol, yeast extract,  $\text{CaCO}_3$  and glycerol as ingredients. The medium has a sugar (D-sorbitol) concentration of generally 1 - 50%, preferably 2 - 40%.

A transformant is cultured at generally pH 5.5 - 8.5, preferably pH 6 - 8, at generally 18 - 40°C, preferably 20 - 35°C for 5 - 150 h.

SLDH can be purified by appropriately combining various separation techniques typically used according to the fraction having an SLDH activity. Since the SLDH of the present invention is NAD(P)<sup>+</sup> dependent, it highly likely localizes in a soluble fraction of the transformant. In this case, after the completion of the culture, the culture is  
10 filtrated or centrifuged to recover the cell, which is then ruptured by ultrasonication, lysozyme treatment, osmotic pressure shock and the like to give a cell extract for use.

When the recombinant SLDH is produced in the aforementioned form wherein a specific amino acid sequence is  
15 added to the terminus, the SLDH can be quickly and easily purified by a treatment including chromatography using a carrier, on which a metal ion chelate capable of adsorbing the specific amino acid sequence is immobilized (immobilized metal affinity chromatography; IMAC). The metal ion chelate  
20 adsorber to be used can be prepared by bringing a solution containing a transition metal (e.g., divalent ion such as cobalt, copper, nickel and iron, trivalent ion such as iron, aluminum and the like, preferably divalent ion of cobalt) into contact with a matrix to which a ligand, for example,  
25 iminodiacetate group, nitrilotriacetate group, tris(carboxymethyl)ethylenediamine group and the like, has

been attached to allow binding with the ligand. The matrix portion of the chelate adsorbent is not particularly limited as long as it is a typical insoluble carrier.

According to the production method of L-sorbose of the present invention, any transformant containing an expression vector containing the above-mentioned DNA encoding SLDH is cultured in a suitable medium, and D-sorbitol is brought into contact with the obtained culture, or, when the SLDH activity is present in an intracellular fraction of the transformant, with a cell extract thereof, to give L-sorbose. The method for bringing D-sorbitol into contact with the culture includes culture of the transformant in a medium containing D-sorbitol.

The present invention also provides a production method of 2KLGA, which utilizes L-sorbose obtained by the above-mentioned method. That is, a host cell capable of converting L-sorbose to 2KLGA, preferably a host cell transformed with an expression vector containing a DNA encoding SDH and a DNA encoding SNDH, is cultured in a suitable medium, and L-sorbose obtained by the above-mentioned method is brought into contact with the obtained culture, or, when the SDH and SLDH activity is present in an intracellular fraction of the host cell, with a cell extract thereof, to give 2KLGA. The method for bringing L-sorbose into contact with the culture includes culture of the host cell in a medium containing L-sorbose.

According to a different production method of 2KLGA of the present invention, a host cell capable of converting L-sorbose to 2KLGA, which is transformed with an expression vector containing a DNA encoding the above-mentioned SLDH, is  
 5 cultured in a suitable medium, and D-sorbitol is brought into contact with the obtained culture, or, when the SLDH, SDH and SLDH activity is present in an intracellular fraction of the host cell, with a cell extract thereof, to give 2KLGA. The method for bringing D-sorbitol into contact with the culture  
 10 includes culture of the host cell in a medium containing D-sorbitol.

The medium and culture conditions to be used for the production method of L-sorbose and the production method of 2KLGA of the present invention may be the same as or  
 15 partially different from those used for the above-mentioned production method of SLDH.

When D-sorbitol or L-sorbose is brought into contact with a cell extract, the culture after the completion of culture is centrifuged or filtrated to recover the cell,  
 20 which is suspended in a suitable buffer, such as acetate buffer, and the cell is ruptured by ultrasonication and the like and subjected to a centrifugation treatment to give a supernatant which can be used as a cell extract.

The L-sorbose or 2KLGA thus produced can be purified  
 25 from a reaction mixture (when the transformant is cultured in a medium containing D-sorbitol or L-sorbose, a culture





lauroylsarcosylate 100 mM EDTA (pH 8.5)] and proteinase K (final concentration 100 µg/ml), and the mixture was stood at 50°C for 2 hours. Thereto were added caesium chloride (5.5 g) and 5 mg/ml ethidium bromide (0.3 ml) and the mixture was ultracentrifuged at 20°C, 50,000 rpm for 16 hours. The part containing a chromosomal DNA was isolated, dissolved in TE buffer [30 ml, 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA] and dialyzed twice against 5 L of 1 mM EDTA. The dialyzate was washed 4 times with isobutanol, twice with phenol and 3 times with chloroform, and purified by ethanol precipitation. This was dissolved in TE buffer (10 ml) to give a 180 µg/ml chromosomal DNA solution.

## (2) Preparation of cosmid library

A single colony of *Escherichia coli* DH1/pcos6EMBL (ATCC 37571; purchased from ATCC through Sumitomo Pharma International Co. Ltd.) was cultured in a 50 µg/ml kanamycin-containing LB medium [3 ml, 1% polypeptone, 0.5% yeast extract, 1% sodium chloride (pH 7.4)] at 37°C for 16 hours, and 0.5 ml thereof was inoculated to 50 µg/ml kanamycin-containing LB medium (50 ml) in a 500 ml Erlenmeyer flask. The medium was cultured at 37°C for 8 hours and the cells were harvested by centrifugation (6,000 rpm, 10 minutes). The cosmid pcos6EMBL was purified with QIAGEN Plasmid Midi Kit (QIAGEN). The pcos6EMBL (25 µg) was digested with 50 U BamHI at 37°C for 2 hours, and purified by ethanol

precipitation. This was subjected to dephosphorylation with 3 U calf intestine-derived alkaline phosphatase (CIAP) at 37°C for 1 hour and purified by ethanol precipitation.

Separately, the chromosomal DNA (100 µg) of the strain *G.*

5 *oxydans* G624 obtained in the above-mentioned (1) was

partially digested with 5 U Sau3AI at 37°C for 1 minute, and purified by ethanol precipitation. The partial digest (ca.

1.5 µg) and BamHI digest of pcos6EMBL (ca. 3 µg) were ligated with 3 U T4 DNA ligase at 4°C for 16 hours. A portion (3 µl)

10 thereof was subjected to *in vitro* packaging using GIGAPACK II Gold Packaging Extract (STRATAGENE). This packaging solution

was diluted 50-fold with SM buffer [50 mM Tris-HCl (pH 7.5) - 100 mM NaCl - 8 mM MgSO<sub>4</sub> - 0.1% gelatin] and 25 µl of the

indicating bacteria (*Escherichia coli* XL1-Blue MRA) was

15 infected with 25 µl thereof, sown on a 50 µg/ml kanamycin-containing LB plate and stood at 37°C overnight. About 400 colonies were obtained, which means a cosmid library of about 400000 clones was obtained.

### (3) Screening of clone having SLDH activity

20 In a 96 well plate rounded bottom (Nalge) containing a

0.9-fold diluted LB medium containing 5% sorbitol and 50

µg/ml kanamycin by 150 µl per well, 368 cosmid clones were

cultured with gentle shaking at 30°C for 3 days. After

centrifugation (2,000 rpm, 10 minutes), 0.5 mg/ml resorcin-

25 ethanol solution (30 µl) and 0.216 mg/ml ferric sulfate (III)

ammonium-hydrochloric acid solution (30  $\mu$ l) were added to the culture supernatant (20  $\mu$ l), and the mixture was heated at 80°C for 1 hour. Using a medium alone similarly reacted as a control, 3 clones of 1A4, 1A5, 4A9 which showed deeper brown color than did the control were selected as the clones having a conversion capability to sorbose (fructose). The culture supernatants thereof were analyzed by HPLC [column: Polyspher OA KC (E. Merck), 7.8×300 mm; temperature: room temperature; migration phase: 0.01N H<sub>2</sub> SO<sub>4</sub> ; flow amount: 0.4 ml/minute; detection: RI] and sorbose was detected for each clone. Thus, these 3 clones were considered to have an SLDH activity. The length of the insert part of these cosmid clones was about 40 kb for all of them.

#### (4) Subcloning to charomid vector (downsizing of insert)

The cosmid clone 1A4 (300 ng) having an SLDH activity was partially digested with 20 mU Sau3AI at 37°C for 1 hour. The charomid 9-28 (1  $\mu$ g, Nippon Gene) was digested with 4 U BamHI at 37°C for 1 hour. These two solutions were mixed, purified by ethanol precipitation, dissolved in 2-fold diluted TE buffer (5  $\mu$ l) and ligated with 1 U T4 DNA ligase at 4°C for 16 hours. One(1)  $\mu$ l thereof was subjected to *in vitro* packaging using GIGAPACK II XL Packaging Extract (STRATAGENE). The packaging solution (75  $\mu$ l) and SM buffer (75  $\mu$ l) were mixed and used for infecting 150  $\mu$ l of indicating bacteria (*Escherichia coli* DH-1), which was sown

on a 50 µg/ml ampicillin-containing LB plate and incubated at 37°C for 1 day. Of the colonies appeared, 95 colonies were cultured with gentle shaking at 30°C for 3 days in a 96 well plate rounded bottom (Nalge) containing a 0.9-fold diluted LB medium containing 5% sorbitol and 50 µg/ml kanamycin by 150 µl per well. After centrifugation (2,000 rpm, 10 minutes), 0.5 mg/ml resorcin-ethanol solution (30 µl) and 0.216 mg/ml ferric sulfate (III) ammonium-hydrochloric acid solution (30 µl) were added to the culture supernatant (20 µl), and the mixture was heated at 80°C for 1 hour. Using a medium alone similarly reacted as a control, 6 clones of G1, C2, A4, B7, H10, B12 which showed deeper brown color than did the control were selected as the clones having a conversion capability to sorbose. The length of the insert part of these charomid clones was about 15 kb for all of them.

(5) Subcloning of SLDH gene to plasmid vector

From the restriction enzyme map of the clones obtained so far, it was found that SLDH gene did not have a SacI site or a XbaI site. Thus, 1 µg of charomid B7 was digested with 10U of SacI and 10U of XbaI to give about 6 kb (B7SX3) and about 9 kb (B7SX2) SacI-XbaI fragments. These two fragments were respectively ligated with *Escherichia coli*-*Pseudomonas* shuttle vector pUCP19 [1.8 kb PstI fragment derived from pRO1614 was inserted into NarI site of pUC19 and purified from *Escherichia coli* DH5 αF' (ATCC 87110)] and transformed

with *Pseudomonas* (this strain was later named *Pseudomonas* sp. F-1, hereinafter to be referred to by this designation) by the electroporation method to give Ps./pUCP19-B7SX3 and Ps./pUCP19-B7SX2. The preparation of competent cell and  
5 conditions of transformation followed those of *Escherichia coli*. These two clones were cultured in a medium containing sorbitol. As a result, sorbose conversion capability was found in Ps./pUCP19-B7SX2. Therefore, Ps./pUCP19-B7SX2 was cultured in a medium (pH 7.4) containing 5% sorbitol, 1%  
10 bactotrypton, 0.5% yeast extract, 1% sodium chloride and 50 µg/ml ampicillin at 30°C for 4 days to give 2.4 mg/ml of sorbose (conversion efficiency: 5%). This sorbose was separated by HPLC and the coincidence of retention time with the standard product was confirmed. HPLC was performed under  
15 the same conditions as in the above-mentioned (3). Using GC/MS [column:DB-5 (J & W Scientific), 0.32 mm × 30 m (film 0.25 µm); temperature: injection=230°C, column=100°C (5 minutes) → heating at 10°C/minute for 10 minute → 200°C (5 minutes) → heating at 30°C/minute for 1 minute → 230°C (4  
20 minutes), detect=230°C; flow amount: pressure control 20 kPa(He)], the coincidence of mass pattern with the standard product was confirmed.

(6) Determination of base sequence of SLDH gene

The restriction enzyme map of the insert part of  
25 plasmid pUCP19-B7SX2 of transformant of *Pseudomonas*,  
Ps./pUCP19-B7SX2, that expresses an SLDH activity was assumed

as shown in FIG. 2. By digestion of 1 µg of pUCP19-B7SX2 with 10U of Hind III at 37°C for 1 hour, about 4 kb Hind III-Hind III fragment was obtained. This Hind III-Hind III fragment was ligated with vector pUCP19 and plasmid pUCP19-HC was constructed. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-HC. This transformant was cultured in a medium containing sorbitol. As a result, expression of SLDH activity was acknowledged. Thus, this Hind III-Hind III fragment was found to contain full length SLDH gene. The base sequence of this about 4 kb Hind III-Hind III fragment was determined. First, the insert part of pUCP19-HC was divided into about 1.1 kb SphI-SphI fragment (S1), about 0.8 kb EcoRI-SphI fragment (ES) and about 1.3 kb EcoRI-EcoRI (E1) fragment (FIG. 2), and each was subcloned to pUC18 to give pUC18-S1, pUC18-ES and pUC18-E1.

Using plasmids pUCP19-HC, pUC18-S1, pUC18-ES and pUC18-E1 as templates and using universal primer and reverse primer (New England Labs.), which were M13 sequencing primers, first sequencing was performed. The sample was fluorescent labeled with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The following 11 kinds of primers were synthesized and using pUCP19-HC as a template sequencing was performed, whereby the base sequence of about 4 kb Hind III-Hind III fragment was determined (Sequence Listing SEQ ID NO:2).

## SLDH gene sequencing primer

	SL1	GCTGCTGAGTGATCCG	(Sequence Listing SEQ ID NO:3)
	SL2	GACTGCTACTTCGATCC	(Sequence Listing SEQ ID NO:4)
	SL3	CCTACACCTAGCCTGC	(Sequence Listing SEQ ID NO:5)
5	SL4	CAGTGCCGTCATGAGG	(Sequence Listing SEQ ID NO:6)
	SL5	TCCTGATCTCGGTGCG	(Sequence Listing SEQ ID NO:7)
	SL6	GATGCTTCAGCACGGC	(Sequence Listing SEQ ID NO:8)
	SL7	GACGATCACGGAAGGC	(Sequence Listing SEQ ID NO:9)
	SL8	GGTTACGTGGTCGAGG	(Sequence Listing SEQ ID NO:10)
10	SL9	CTATACGTGACAGGTCC	(Sequence Listing SEQ ID NO:11)
	SL10	GCGCGATCTGGATACG	(Sequence Listing SEQ ID NO:12)
	SL11	CGAGGATCTCGAACGG	(Sequence Listing SEQ ID NO:13)

From the analysis of the base sequence, 1455 bp ORF was found (base number 537 - 1991). Therefore, SLDH was assumed to consist of 485 amino acids and has a molecular weight of about 54 kDa. As a result of the homology search, it showed 42% homology with mannitol dehydrogenase of *Pseudomonas fluorescence*.

## 20 **Example 2** Production of recombinant SLDH

(1) construction of plasmid expressing SLDH having histidine-tag (hereinafter to be referred to as His-tagged SLDH)

For purification of the recombinant protein, a tag system utilizing 6xhistidine was an extremely easy method. That is, a protein having 6 histidine tag is expressed, and utilizing interaction of a metal (e.g., cobalt, nickel) and





and SacI site (underlined)]

TAGCTGAGCTCATGGGACAGATCTGAGC (Sequence Listing SEQ ID  
NO:17)

The about 360 bp fragment specifically amplified in PCR  
1 was digested with NheI and BamHI, and the about 100 bp  
fragment specifically amplified in PCR 2 was digested with  
BamHI and SacI. Separately, the about 2 kb fragment obtained  
by digesting pUCP19-HC with BglII and PstI was inserted into  
BamHI-PstI fragment of pUCP19 to give plasmid pUCP19-SLDH  
10 (FIG. 3) wherein the downstream of BglII site of the insert  
was removed. This was digested with NheI and SacI, and the  
obtained about 6.2 kb fragment was ligated with the above-  
mentioned two PCR amplification fragments with T4 DNA ligase  
to construct pUCP19-SLDH-His. *Pseudomonas* was transformed  
15 with this plasmid to give Ps./pUCP19-SLDH-His.

## (2) Purification of His-tagged SLDH

One loopful of cryopreservation stock of transformant  
Ps./pUCP19-SLDH-His was inoculated to LB medium (2 ml)  
containing 50 µg/ml ampicillin in a 15 ml centrifuge tube  
20 (Corning) and cultured at 30°C for 16 hours. The 1.5 ml  
thereof was inoculated to LB medium (50 ml) containing 5%  
sorbitol and 50 µg/ml ampicillin in a 500 ml Erlenmeyer flask  
and cultured at 25°C for 3 days. The cells were harvested by  
centrifugation (6,000 rpm, 4°C for 5 minutes), and suspended  
25 in 10 ml of 100 mM NaCl-containing 20 mM Tris-HCl (pH 8.0).  
The suspension was treated with an ultrasonication

homogenizer (Tomy UD-201) for 5 minutes (50% interval),  
centrifuged (15,000 rpm, 4°C for 10 minutes) and a  
supernatant was recovered to give a cell-free extract. TARON  
resin (2 ml, CLONTECH) was placed in a 15 ml centrifuge tube  
5 (Corning), and washed twice with 10 ml of 100 mM NaCl-  
containing 20 mM Tris-HCl (pH 8.0) for equilibration. The  
above-mentioned cell-free extract (5 ml) was added and the  
mixture was shaken at room temperature for 20 minutes to  
adsorb His-tagged SLDH, followed by washing 3 times with 100  
10 mM NaCl-containing 20 mM Tris-HCl (10 ml, pH 8.0) over 10  
minutes. 100 mM NaCl-containing 20 mM Tris-HCl buffers (2  
ml, pH 8.0) respectively containing 10 mM, 30 mM, 50 mM and  
100 mM imidazole were added successively, and shaken at room  
temperature for 2 minutes to elute His-tagged SLDH. As a  
15 result, SLDH activity eluted in a 30 mM - 50 mM imidazole  
fraction. This fraction was applied to SDS-PAGE analyze to  
detect nearly single band.

### (3) Analysis of N terminus amino acid sequence

The His-tagged SLDH purified in the above-mentioned (2)  
20 was electrophoresed using a multigel (Daiichi Pure Chemicals)  
having a gel concentration of 12.5% with 40 mA current over 1  
hour, and using Horiz-Blot (Atto), transferred onto a PVDF  
membrane (Immobilon PSQ; Millipore). The membrane was  
stained with coomassie brilliant blue G-250, and a band  
25 seemingly an about 55 kDa SLDH was cut out with a pair of  
scissors. This PVDF membrane was subjected to amino acid

sequence analysis using protein sequencer G100A (Hewlett-Packard) and PTH analyzer 1090 (Hewlett-Packard). As a result, a sequence (MITRETLKSL; Sequence Listing SEQ ID NO:18) consistent with N terminus amino acid sequence expected from ORF of SLDH gene was obtained.

#### (4) Confirmation of SLDH activity

In the same manner as in the above-mentioned (2) except that the cell-free extract to be applied was by 10 ml, the resin after His-tagged SLDH adsorption was washed 6 times, and His-tagged SLDH was eluted with 50 mM imidazole and 100 mM NaCl-containing 20 mM Tris-HCl (5 ml, pH 8.0), His-tagged SLDH was purified. The obtained His-tagged SLDH was reacted with sorbitol and the resulting product was analyzed. The composition of the reaction solution (2 ml) was 10 mM (1.82 mg/ml) sorbitol, 0.1 M glycine/NaOH buffer (pH 10.1), 5 mM NADP<sup>+</sup> and His-tagged SLDH 0.2 ml (41.4 µg protein) and the reaction was carried out at 25°C for 24 hours. As a result, 1.12 mg/ml sorbose was generated (sorbitol remaining in 0.70 mg/ml; conversion efficiency: 62%). Thus, His-tagged SLDH purified by cobalt type IMAC was confirmed to be sorbitol dehydrogenase that oxidizes sorbitol and generates sorbose.

### Example 3 Characterization of SLDH

#### (1) Coenzyme dependency and active pH range

To a solution containing 0.1 ml of 50 mM NAD<sup>+</sup> (or NADP<sup>+</sup>), 0.2 ml of 500 mM buffer, 10 µl of His-tagged SLDH solution

(2.1  $\mu$ g protein) prepared in Example 2(4) and distilled water (0.29 ml) was added to 500 mM sorbitol (0.4 ml) to start the reaction (25°C), and increase in NADH (or NADPH) was measured by a spectrophotometer (UV-2200; Shimadzu) based on absorbance at 340 nm as an index. For the reaction solutions having pH 10.1 and pH 9.0, glycine/NaOH buffer was used, and for reaction solutions having pH 8.0 and pH 7.0, potassium phosphate buffer was used. The enzyme activity (1 unit) was defined to be an amount to generate 1  $\mu$ mol of NADH (or NADPH) per minute. The molecular extinction coefficient of NAD(P)H was 6.3  $\text{mM}^{-1} \text{cm}^{-1}$ . The protein amount was measured with bovine serum albumin (BSA) as a standard by the Lowry method. As a result, SLDH could utilize both  $\text{NAD}^+$  and  $\text{NADP}^+$  as coenzymes, and  $\text{NADP}^+$  showed higher specificity. The activity of this enzyme was higher in the alkaline pH (Table 1).

Table 1

Coenzyme	pH	Activity (U/mg protein)
$\text{NADP}^+$	10.1	130.2
	9.0	30.0
	8.0	22.9
	7.0	4.2

NAD <sup>+</sup>	10.1	8.1
	9.0	3.4
	8.0	1.2
	7.0	0.1

## (2) Substrate specificity

In the same manner as in the above-mentioned (1) except that the reaction solution contained various substrates to replace sorbitol, the buffer was glycine/NaOH buffer (pH 10.1), and coenzyme was NADP<sup>+</sup>, the SLDH activity was measured. As a result, this enzyme could utilize, besides sorbitol, mannitol and arabitol as a substrate, but showed no action on xylitol, ribitol, inositol or glycerol (Table 2).

10

Table 2

Substrate	Activity (U/mg protein)
sorbitol	130.2
mannitol	85.7
arabitol	88.1
xylitol	0
ribitol	0
inositol	0
glycerol	0

## (3) Michaelis constant

Using sorbitol as a substrate, SLDH activity was measured according to the method of the above-mentioned (2). As a result, the  $K_m$  value for sorbitol was 132 mM (25°C).

5 **Example 4** Preparation of *Pseudomonas* transformant having SNDH/SDH expression vector and study of 2KLGA productivity by this transformant

Of the pBBR plasmids that are broad host range plasmids [Gene, 166, 175 (1995)] supplied by Dr. Kovach at Louisiana State University, Medical Center, SNDH/SDH gene was introduced into the genus *Pseudomonas* strain using pBBR1MCS-2 (kanamycin resistant) and pBBR1MCS-3 (tetracycline resistant) as vectors, and fermentative production of 2KLGA from L-sorbose by the obtained transformant was studied.

15 (1) Construction of SNDH/SDH expressing broad host range plasmid

Plasmid pSDH-tufB1-Eco-d9U (FIG. 4) (5 µg) containing SNDH/SDH gene and using tufB as a promoter was digested with EcoRI (50U, Behringer-Mannheim) at 37°C for 1 hour, and electrophoresed on 0.8% agarose gel, which was followed by separation of a 3.7 kb EcoRI/EcoRI fragment containing SNDH/SDH gene. This fragment was inserted into the EcoRI site of pBBR1MCS-2. The plasmid inserted in the same direction as the  $\beta$ -galactosidase gene was taken as pBBR (Km) - SDH SNDH (FIG. 5).

Plasmid pSDH-tufB1 (10 µg, construction method is





### (3) Transformation of *Pseudomonas*

The competent cells of *Pseudomonas* cryopreserved in liquid nitrogen sp.F-1 were thawed in ice water, and solutions of an SNDH/SDH expressing broad host range plasmid constructed in the above-mentioned (1), pBBR(Km)-SDH· SNDH and pBBR(Tc)-SDH· SNDH, were added by 1  $\mu$ l (ca. 1  $\mu$ g) each, and stood at 4°C for 30 minutes. This was transformed using a Gene Pulser gene transfer device (Bio-Rad) in a cuvette having a distance of 0.1 cm between electrodes under the conditions of 200 $\Omega$ , 1.8 kV, 25  $\mu$ F, and suspended in L medium containing 0.4% glucose, which was followed by shaking at 30°C for 1 hour. They were sown on an L agar plate containing 50  $\mu$ g/ml kanamycin and an L agar plate containing 20  $\mu$ g/ml tetracycline, cultured at 30°C for 2 days to give transformant Ps./pBBR(Km)-SDH· SNDH and Ps./pBBR(Tc)-SDH· SNDH.

### (4) Fermentative production of 2KLGA from sorbose by the transformant

A single colony of transformants Ps./pBBR(Km)-SDH· SNDH and Ps./pBBR (Tc)-SDH· SNDH obtained in the above-mentioned (3) was each inoculated to 5 ml of L medium in a 16.5×165 mm test tube and cultured at 30°C for 2 days. The culture solution (0.5 ml) was inoculated to a medium (10 ml, pH 7.4) for 2KLGA production containing 5% sorbose, 0.1% glucose, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9%

sodium chloride and 2% calcium carbonate in a 100 ml Erlenmeyer flask, and cultured at 30°C for 5 days. The culture solution was separated by centrifugation and sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were quantitatively determined. The sorbose, sorbosone, 2KLGA and L-idonic acid were each quantitatively determined by HPLC under the following conditions.

[sorbose]

column: Polyspher OA KC (7.8 mm inner diameter×300 mm;

10                   Cica-MERCK)

migration phase: 0.01N H<sub>2</sub>SO<sub>4</sub>

column temperature: room temperature

flow rate: 0.4 ml/minute

detection: differential refractometer

```
15 [sorbosone (post-column labeling method)]
```

column: Polyspher OA KC (7.8 mm inner diameterx300 mm; Cica-MERCK)

migration phase (labeling agent): 0.04M benzamidine

hydrochloride

20                      0.25M potassium sulfite

2 mM boric acid/0.1N potassium hydroxide

flow rate: 0.3 ml/minute

detection: fluorescent detector (excitation wavelength: 315 nm, detection wavelength: 405 nm)

25 [2KLGA and L-idonic acid]

column: Capcell pak NH2 (4.6 mm inner diameter×250 mm;



For comparison, production of 2KLGA and L-idonic acid by a non-transformant *Pseudomonas* sp. F-1 was also investigated. The glycerol cryopreserved cells of *Pseudomonas* sp. F-1 were inoculated to 5 ml of L medium in a 16.5x165 mm test tube and cultured at 30°C for 1 day. The culture solution (1 ml) was inoculated to a medium (10 ml, pH 7.4) containing 5% sorbose, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco) and 0.9% sodium chloride in a 100 ml Erlenmeyer flask, and cultured at 30°C for 3 days. The culture solution was centrifuged and sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were similarly determined quantitatively. As a result, sorbose was consumed (5.7 mg/ml) but sorbosone, 2KLGA and L-idonic acid were not detected.

From the above, by the introduction of SNDH/SDH gene into 2KLGA and L-idonic acid non-producing *Pseudomonas* sp. F-1, a transformant that highly produces 2KLGA and L-idonic acid from sorbose could be obtained.

**Example 5** Preparation of *Pseudomonas* transformant containing SNDH/SDH expression vector and consideration of 2KLGA productivity of the transformant - (2)

In the same manner as in Example 4 and using a different strain [strain *Pseudomonas* IF03309; supplied by the Institute for Fermentation, Osaka (17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka)] belonging to the genus

*Pseudomonas* as a host, a transformant was prepared, into which the SNDH/SDH gene was introduced, and the 2KLGA and L-idonic acid productivity of this transformant was investigated.

5 (1) Introduction of SNDH/SDH gene into strain *Pseudomonas* IFO3309

Glycerol cryopreserved cells of the strain *Pseudomonas* IFO3309 were treated in the same manner as in Example 4(2) to prepare cryopreserved cells of competent cells. The  
10 competent cells cryopreserved in liquid nitrogen were thawed in ice water, and a solution 1  $\mu$ l (ca. 1  $\mu$ g) of Ps./pBBR(Km)-SDH<sup>+</sup> SNDH, which was an SNDH/SDH expressing broad host range plasmid, was added and the mixture was stood at 4°C for 30 minutes. This was transformed using a Gene Pulser gene  
15 transfer device (Bio-Rad) under the same conditions as in Example 4(3) to give transformant Ps. IFO3309/pBBR(Km)-SDH<sup>+</sup> SNDH.

(2) Fermentative production of 2KLGA by the transformant

One loopful of the Ps. IFO3309/pBBR(Km)-SDH<sup>+</sup> SNDH  
20 obtained in the above (1) was inoculated to a medium (5 ml, pH 7.0) containing 2% sorbitol and 0.5% yeast extract (Difco) in a 16.5x165 mm test tube, and cultured at 28°C for 1 day. The culture solution (1 ml) was inoculated to a medium (10 ml, pH 7.0) containing 5% sorbitol, 0.5% yeast extract  
25 (Difco), 0.2% polypeptone (Wako Pure Chemical Industries), 0.1% K<sub>2</sub> HPO<sub>4</sub>, 0.5% MgSO<sub>4</sub> · 7H<sub>2</sub>O and 2%CaCO<sub>3</sub> in a 100 ml

Erlenmeyer flask and cultured at 28°C for 7 days. The culture solution was centrifuged and in the same manner as in Example 4(4), sorbitol, sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were quantitatively  
 5 determined. For comparison, non-transformant the strain *Pseudomonas* IFO3309 was cultured under the same conditions and sorbitol, sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were quantitatively determined.

As a result, by the non-transformant, sorbitol was  
 10 consumed (0.4 mg/ml), sorbose was produced (3.9 mg/ml) but sorbosone, 2KLGA and L-idonic acid were not detected. On the other hand, 2KLGA (1.2 mg/ml) and L-idonic acid (0.5 mg/ml) were produced by the transformant Ps. IFO3309/pBBR(Km) - SDH: SNDH (Table 4). In other words, it was confirmed that,  
 15 by the introduction of SNDH/SDH gene into this host, the ability to produce 2KLGA and L-idonic acid from sorbitol was imparted even under the conditions where *Pseudomonas* IFO3309 cannot produce 2KLGA or L-idonic acid.

Table 4

culture results by transformant (mg/ml)

transformant	sorbose	sorbosone	2KLGA	L-idonic acid
<hr/>				
<i>Ps.</i> 3309/pBBR(Km) -				
SDH <sup>+</sup> SNDH	0.41	1.8	1.2	
0.5				

**Example 6** Production of 2KLGA by *Pseudomonas* transformant  
 5 into which SLDH expression vector and SNDH/SDH expression  
 vector were introduced

(1) Preparation of *Pseudomonas* transformant having SLDH  
 expression vector and SNDH/SDH expression vector

As mentioned above, an expression vector pBBR(Km) -  
 10 SDH<sup>+</sup> SNDH (FIG. 5) was constructed by incorporating an  
 SNDH/SDH gene derived from *G. oxydans* T-100 (FERM BP-4188;  
 European Patent Publication EP 0758679 A1) into pBBR1MCS-2.  
 pBBR(Km) -SDH<sup>+</sup> SNDH was introduced into recombinant *Pseudomonas*  
*Ps.*/pUCP19-B7SX2 obtained in Example 1(5) by the  
 15 electroporation method to give *Ps.*/pUCP19-B7SX2+pBBR(Km) -  
 SDH<sup>+</sup> SNDH.

(2) Production of 2KLGA by *Pseudomonas* transformant

*Ps.*/pUCP19-B7SX2+pBBR(Km) -SDH<sup>+</sup> SNDH was cultured in a  
 medium (pH 7.4) containing 5% sorbitol, 1% bactotrypton  
 20 (Difco), 0.5% yeast extract (Difco), 1% sodium chloride, 50  
 µg/ml ampicillin, 50 µg/ml kanamycin and 2% light calcium

carbonate at 30°C for 4 days to give 1.1 mg/ml of 2KLGA and 1.7 mg/ml of idonic acid. The 2KLGA was separated by HPLC and coincidence with a standard product in the retention time was confirmed. Using GC/MS, coincidence with a standard product in the mass pattern was confirmed. HPLC and GC/MS were performed under the same conditions as in Example 1(3) and (5).

**Example 7** Preparation of various *Pseudomonas* transformants

(1) Ps./pUCP19-SLDH+pBBR(Km)-SDH• SNDH

*Pseudomonas* sp.F-1 was transformed with pUCP19-SLDH constructed in Example 2(1) to give Ps./pUCP19-SLDH. pBBR(Km)-SDH• SNDH was further introduced into this recombinant *Pseudomonas* to give Ps./pUCP19-SLDH+pBBR(Km)-SDH• SNDH.

(2) Ps./pUCP19-SLDH-tufB+pBBR(Km)-SDH• SNDH

To introduce SspI site into the upstream of initiation codon of SLDH gene, PCR was performed using pUCP19-SLDH (5 µg) as a template in the presence of the following primers (20 pmol each) using pfu DNA polymerase (2.5 U) (94°C, 30 seconds→55°C, 2 minutes→72°C, 2 minutes, 25 cycles).

sense primer [including SspI site (underlined) and sequence identical with 5' terminus of SLDH coding sequence]

TAGGAATATTTCTCATGATTACGCGCGAAACCC (Sequence Listing SEQ

ID NO:19)

antisense primer [sequence identical with sequence



downstream of EagI site in SLDH coding sequence]

GATGCTTCAGCACGGC (Sequence Listing SEQ ID NO:20)

The about 360 bp fragment specifically amplified by PCR was digested with SspI and EagI. pUCP19-SLDH was digested with PstI and EagI to give about 5.7 kb fragment. These two fragments and PstI-SspI fragment (Sequence Listing SEQ ID NO:21) containing tufB promoter were ligated with T4 DNA ligase to construct pUCP19-SLDH-tufB. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-SLDH-tufB. Furthermore, pBBR(Km)-SDH<sup>+</sup> SNDH capable of expressing SNDH/SDH activity was introduced to give Ps./pUCP19-SLDH-tufB+pBBR(Km)-SDH<sup>+</sup> SNDH.

(3) Ps./pUCP19-3DH

pUCP19-SLDH-tufB (5 µg) was digested with 40 U KpnI and 40 U PstI at 37°C for 1 hour to give a 1.6 kb fragment. pUCP19-SDH<sup>+</sup> SNDH (expression vector obtained by incorporating SNDH/SDH gene derived from *G. oxydans* T-100 into pUCP19; FIG. 7) (1 µg) was digested with 10 U KpnI and 10 U PstI at 37°C for 1 hour to give a 8.2 kb fragment. These two fragments were ligated with T4 DNA ligase to construct pUCP19-3DH. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-3DH.

#### Example 8 Consideration of productivity of 2KLGA

Since production of 2KLGA by recombinant *Pseudomonas* was confirmed, the productivity of 2KLGA was studied in media



[culture 5]

The cells were cultured in the same manner as in [culture 1] except that Ps./pUCP19-SLDH-tufB+pBBR(Km) - SDH SNDH was used as the producing bacteria and 5% glycerol was further added to the main culture medium.

[culture 6]

The cells were cultured in the same manner as in [culture 1] except that Ps./pUCP19-3DH was used as the producing bacteria, kanamycin was removed from the pre-culture medium and the main culture medium and 5% glycerol was further added to the main culture medium.

The sorbitol, sorbose, sorbosone and 2KLGA in each culture were quantitatively determined. The results are shown in Table 5. The propensity toward increased conversion efficiency to 2KLGA was observed by the addition of glycerol to the medium.

[culture 7]

The cells were cultured for 7 days in the same manner as in [culture 1] except that the yeast extract concentration of the main culture medium was set to 2% and 5% glycerol was further added to the main culture medium. The sorbitol, sorbose, sorbosone and 2KLGA at day 1, 3, 5 and 7 of culture were quantitatively determined. The results are shown in Table 5.

culture		sorbitol	sorbose	sorbose	2KLGA	idonic acid
1		44.1	6.3	0.1	3.7	1.6
2		44.8	3.1	0	4.8	2.2
3		26.7	5.1	0	10.9	8.4
4		26.6	0	0	9.0	ND
5		26.6	0	0	10.7	ND
6		30.7	5.2	0	7.5	ND
7	days	sorbitol	sorbose	sorbose	2KLGA	idonic acid
	1	41.1	0	0	4.2	ND
	3	25.6	0	0	10.6	ND
	5	14.2	0	0	16.3	ND
	7	7.6	0	0	18.4	15.5

(unit: mg/ml)

ND: Not determined

5 **Example 9** Fermentative production of sorbose or 2KLGA by *Pseudomonas putida* transformant into which SLDH expression vector and/or SNDH/SDH expression vector were introduced

In the following test, the preparation and transformation of competent cells of the strain *Pseudomonas putida* IF03738 followed the above-mentioned *Pseudomonas* sp.F-1. Because the strain *Pseudomonas putida* IF03738 is ampicillin resistant, when ampicillin resistance is used as a

selection marker, cells were sown on an L agar plate containing 500 µg/ml ampicillin (10-fold amount of normal level) after electroporation, and cultured at 30°C for 1 day to pick up large colonies for the selection of transformant.

- 5 (1) Fermentative production of sorbose from sorbitol by transformant into which SLDH expression vector was introduced

SLDH gene (pUCP19-SLDH) was introduced into the strain *Pseudomonas putida* IF03738. A single colony of the obtained transformant *Pseudomonas putida* IF03738/pUCP19-SLDH was  
 10 inoculated to a medium (10 ml, pH 7.4) for sorbose production, which contained 5% sorbitol, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9% sodium chloride and 500 µg/ml ampicillin in a 100 ml Erlenmeyer flask and cultured at 30°C for 3 days. The culture solution was  
 15 centrifuged and sorbitol and sorbose in the culture supernatant were quantitatively determined. As a result, 34.6 mg/ml of sorbitol remained and 7.6 mg/ml of sorbose was generated.

- (2) Fermentative production of 2KLGA from sorbose by  
 20 transformant into which SNDH/SDH expression vector was introduced

SNDH/SDH gene (pBBR(Km)-SDH·SNDH) was introduced into *Pseudomonas putida* IF03738. A single colony of the obtained transformant *Pseudomonas putida* IF03738/pBBR(Km)-SDH·SNDH was  
 25 inoculated to a medium (10 ml, pH 7.4) for 2KLGA production, which contained 5% sorbose, 0.9% bactotrypton (Difco), 0.45%

yeast extract (Difco), 0.9% sodium chloride, 2% calcium carbonate and 50 µg/ml kanamycin in a 100 ml Erlenmeyer flask and cultured at 30°C for 7 days. The culture solution was centrifuged and sorbose, 2KLGA and idonic acid in the culture supernatant were quantitatively determined. As a result, 34.3 mg/ml of sorbose remained and 13.9 mg/ml of 2KLGA and 3.5 mg/ml of idonic acid were generated.

(3) Fermentative production of 2KLGA from sorbitol by transformant into which SLDH expression vector and SNDH/SDH expression vector were introduced

SLDH and SNDH/SDH genes (pUCP19-SLDH and pBBR(Km)-SDH~~+~~ SNDH) were introduced into the strain *Pseudomonas putida* IF03738. A single colony of the obtained transformant *Pseudomonas putida* IF03738/pUCP19-SLDH+pBBR(Km)-SDH~~+~~ SNDH was inoculated to a medium (10 ml, pH 7.4) for 2KLGA production, which contained 5% sorbitol, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9% sodium chloride, 2% calcium carbonate, 500 µg/ml ampicillin and 50 µg/ml kanamycin in a 100 ml Erlenmeyer flask and cultured at 30°C for 7 days. The culture solution was centrifuged and sorbitol, sorbose, 2KLGA and idonic acid in the culture supernatant were quantitatively determined. As a result, 35.6 mg/ml of sorbitol remained and 13.2 mg/ml of 2KLGA and 6.2 mg/ml of idonic acid were generated. Sorbose was not detected.

25

**Free text of Sequence Listing**

- SEQ ID NO:3: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:4: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- 5 SEQ ID NO:5: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:6: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:7: Oligonucleotide designed to act as primer for  
10 sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:8: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:9: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- 15 SEQ ID NO:10: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:11: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:12: Oligonucleotide designed to act as primer for  
20 sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:13: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:14: Oligonucleotide designed to act as sense primer for amplifying DNA sequence encoding His-tagged SLDH and  
25 promoter.
- SEQ ID NO:15: Oligonucleotide designed to act as antisense





[illegible]

1. A sorbitol dehydrogenase having the following physicochemical properties:

5 L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme:  $\text{NAD(P)}^+$  dependent

10 inositol or glycerol.

2. The sorbitol dehydrogenase of claim 1, which is derived from the strain *Gluconobacter oxydans* G624.

15 3. A sorbitol dehydrogenase which is originated from the same  
gene as is the sorbitol dehydrogenase of claim 2 in its  
molecular evolution.

20 from a bacteria belonging to the genus *Gluconobacter*.

5.. A sorbitol dehydrogenase which is the following protein

(a) or (b) :

25 in Sequence Listing SEQ ID NO:1

(b) a protein consisting of the same amino acid sequence as



11. A promoter gene comprising the DNA of the following (a)  
or (b):

(a) a DNA having a base sequence of base numbers 1 - 536 of  
the base sequence depicted in Sequence Listing SEQ ID NO:2

5 (b) a DNA having a base sequence of the above-mentioned (a)  
wherein one to several bases is(are) deleted, substituted,  
inserted, added or modified, which DNA shows a promoter  
activity at least in one kind of microorganism.

10 12. A recombinant vector comprising a DNA of any of claims 6  
to 9.

13. An expression vector comprising a DNA of any of claims 6  
to 9.

15

14. The expression vector of claim 13 further comprising a  
DNA encoding sorbose dehydrogenase and/or a DNA encoding  
sorbosone dehydrogenase.

20 15. A transformant obtained by transforming a host cell with  
an expression vector of claim 13 or 14.

16. The transformant of claim 15, which belongs to a genus  
selected from the group consisting of *Escherichia coli*, the  
25 genus *Pseudomonas*, the genus *Gluconobacter*, the genus  
*Acetobacter* and the genus *Pseudogluconobacter*.

17. The transformant of claim 15 or 16, which is capable of converting D-sorbitol to 2-keto-L-gulonic acid.

5 18. A method for producing a protein having a sorbitol  
dehydrogenase activity, which method comprises culturing a  
host cell transformed with an expression vector of claim 13  
in a medium and harvesting the sorbitol dehydrogenase of any  
of claims 1 to 5 or the protein of claim 10 from the obtained  
10 culture.

19. A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium and bringing D-  
15 sorbitol into contact with the obtained culture or a treated product thereof.

20. A method for producing 2-keto-L-gulonic acid, which method comprises culturing a host cell transformed with an expression vector containing a DNA encoding sorbose dehydrogenase and a DNA encoding sorbosone dehydrogenase in a medium and bringing the L-sorbose obtained according to the method of claim 19 into contact with the obtained culture or a treated product thereof.

25

21. A method for producing 2-keto-L-gulonic acid, which

method comprises culturing the transformant of claim 17 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

- 5 22. A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gulonic acid obtained by the method of claim 20 or 21 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

## ABSTRACT OF THE DISCLOSURE

The present invention provide a gene encoding D-sorbitol dehydrogenase (SLDH), a method for producing SLDH by culture of a host cell transformed with a vector expressing this gene and a method for producing L-sorbose or 2-keto-L-gulonic acid (2KLGA) using the culture. 2KLGA is an important intermediate for the production of L-ascorbic acid. Therefore, the present invention also provides a method for producing L-ascorbic acid from the 2KLGA obtained by the above-mentioned method.

FIG. 1

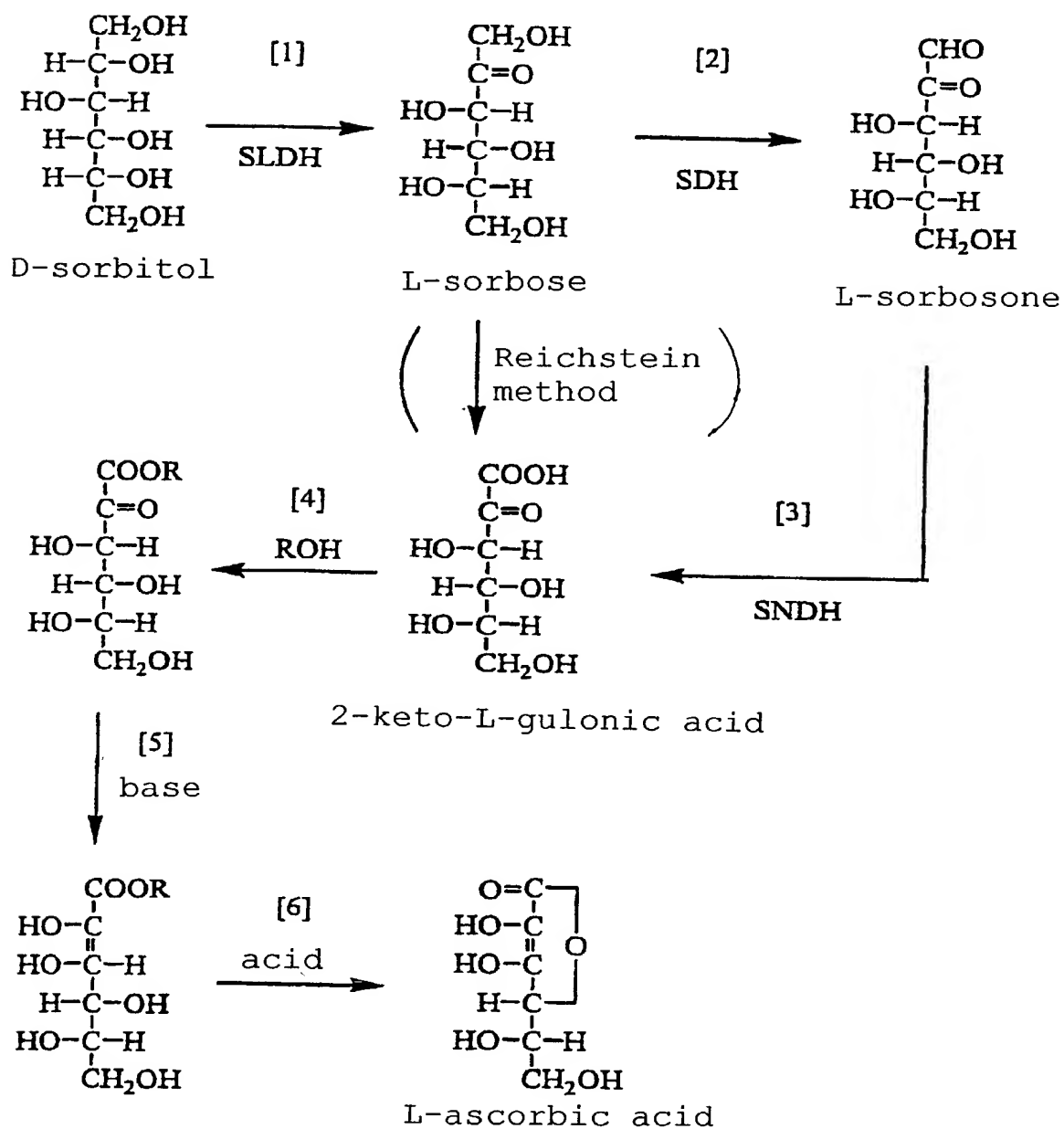


FIG. 2

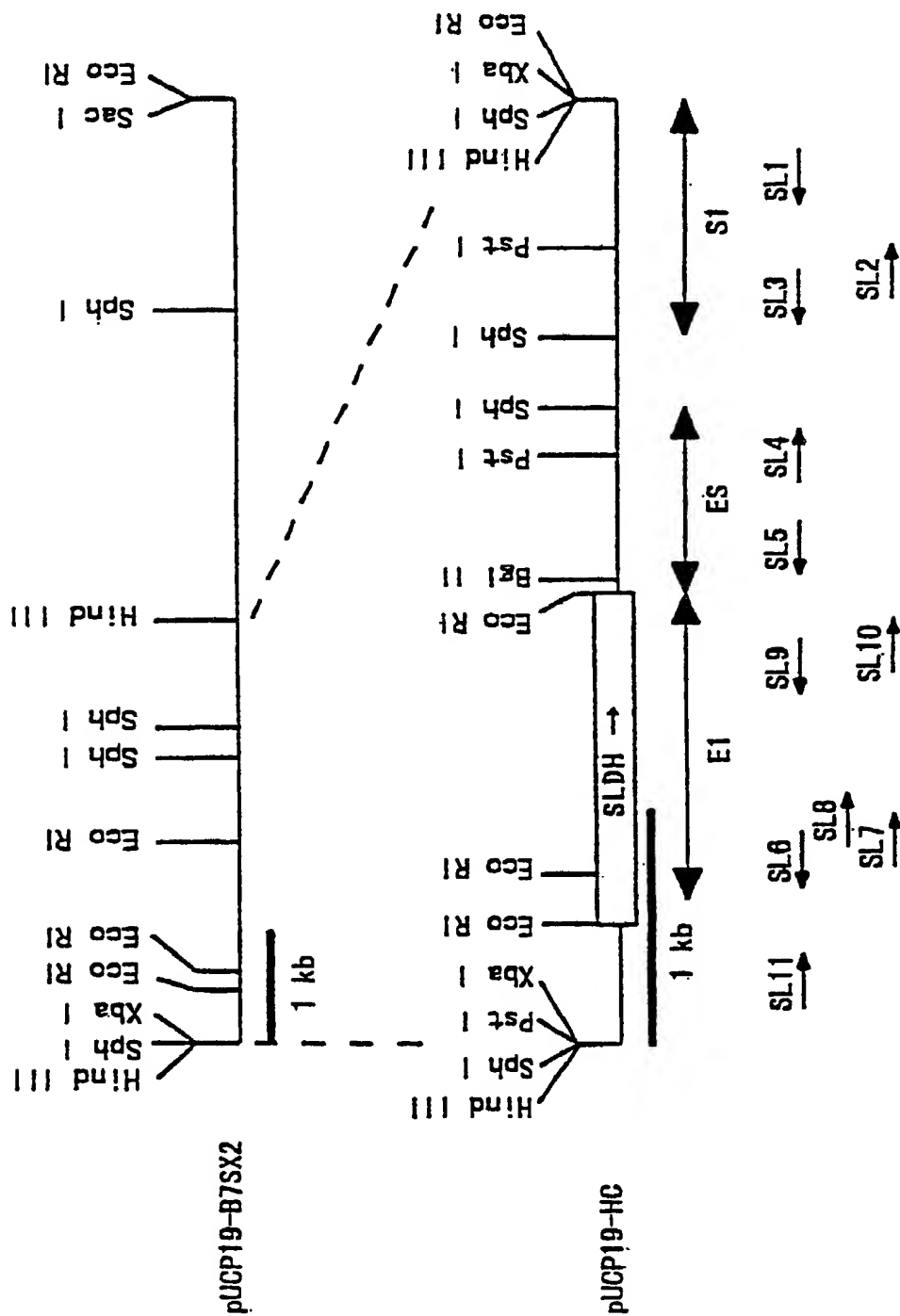
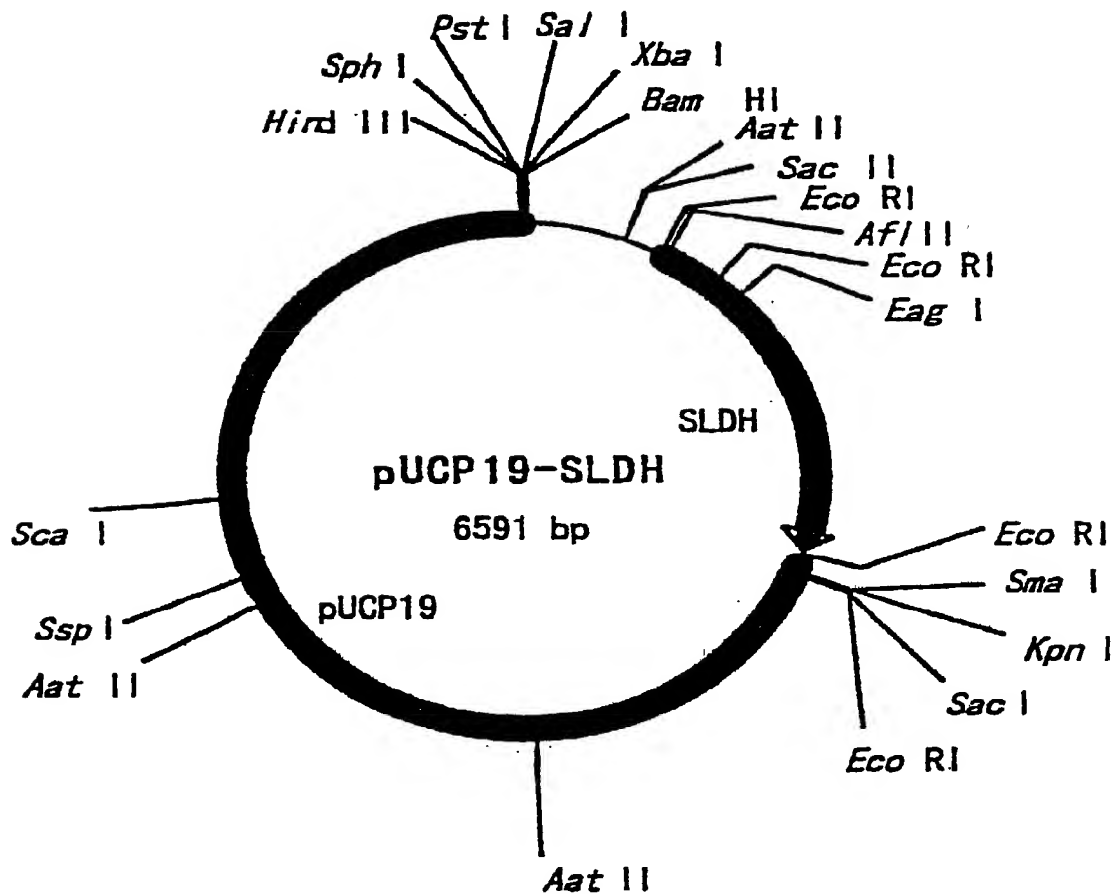




FIG. 3



**FIG. 4**

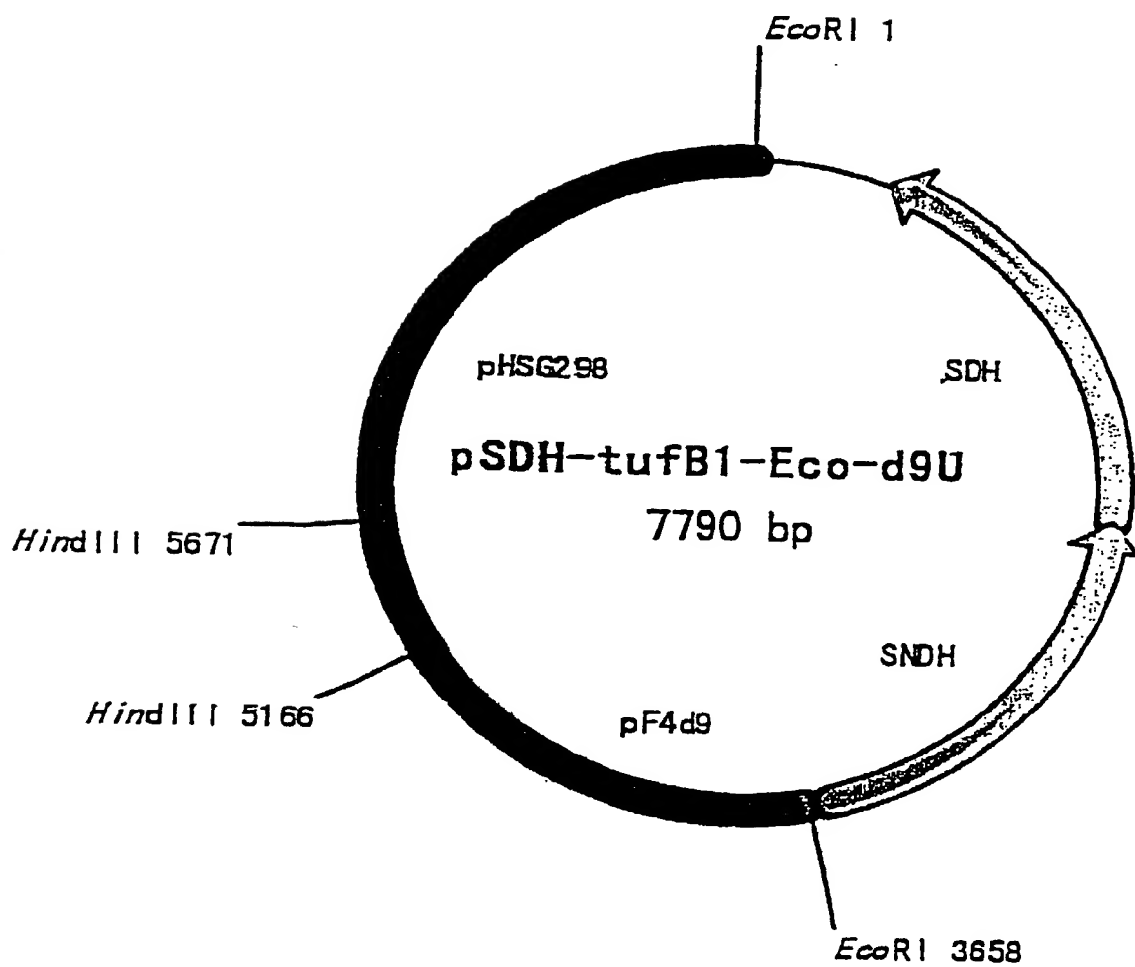


FIG. 5

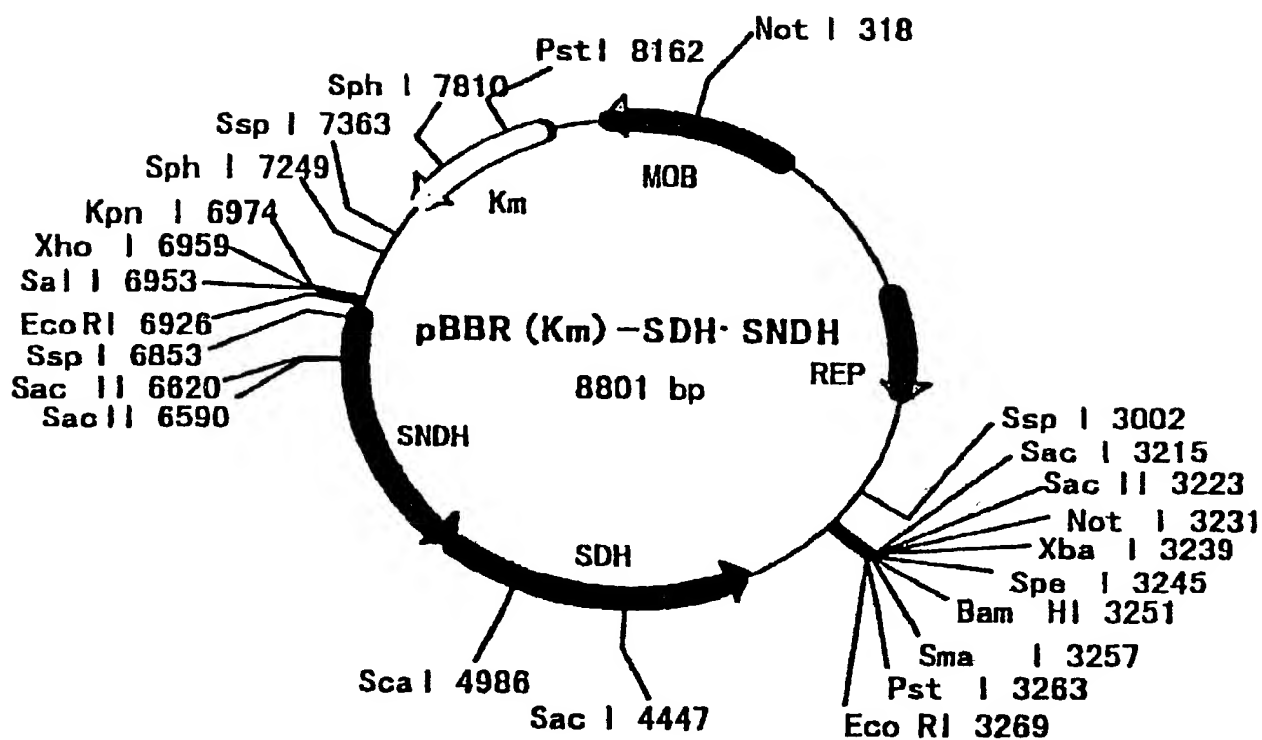


FIG. 6

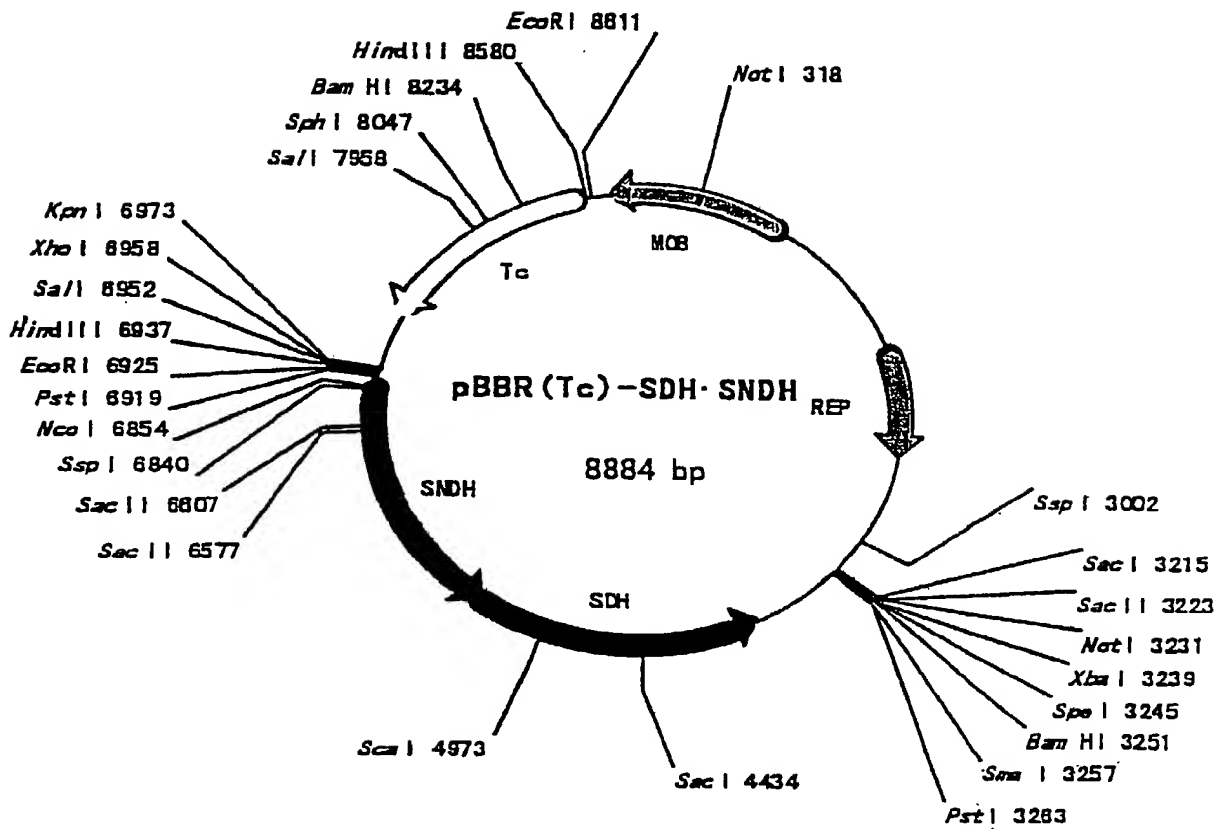
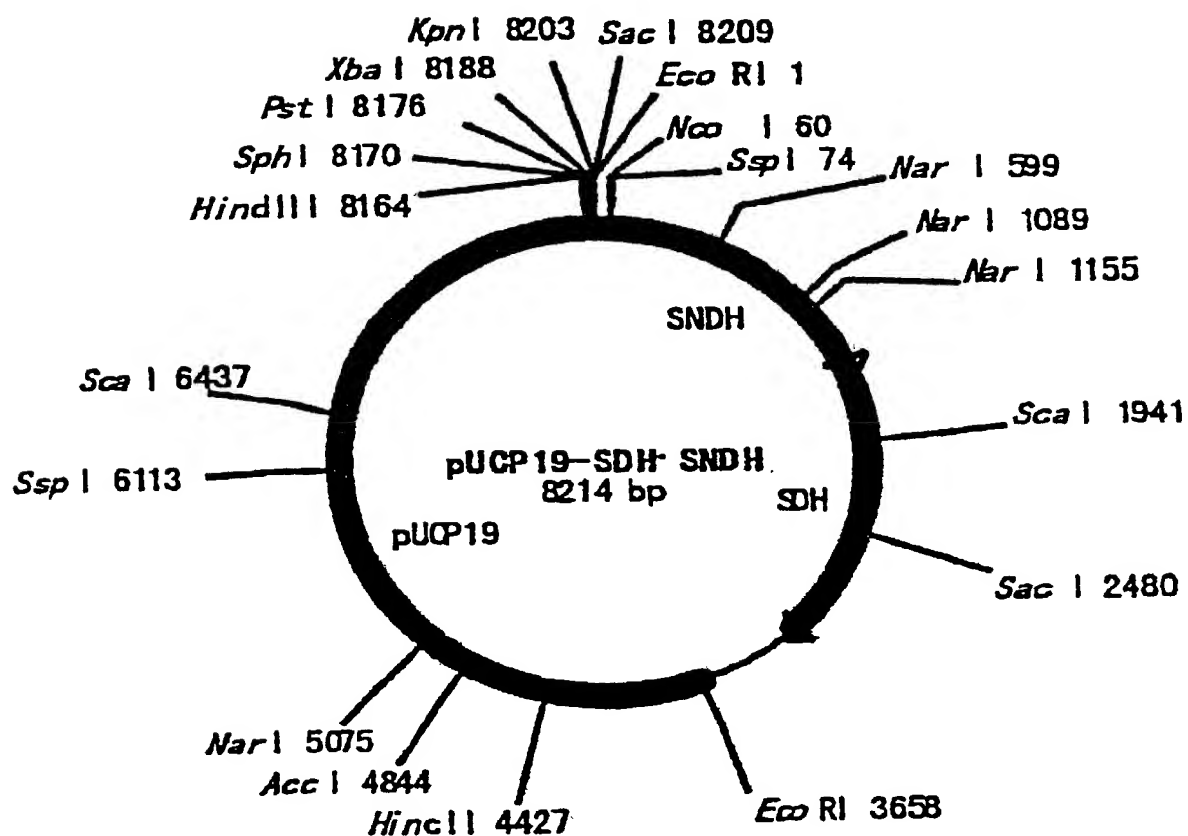


FIG. 7



# Declaration, Power of Attorney and Petition

Page 1 of 4

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE THEREOF

the specification of which

☐ is attached hereto.

☒ was filed on September 17, 2001 as  
Application Serial No. 09/926,163  
and amended on \_\_\_\_\_.

☒ was filed as PCT international application

Number PCT/JP00/01608

on March 16, 2000,

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b,) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
<u>72810/1999</u>	<u>Japan</u>	<u>17/03/1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>224679/1999</u>	<u>Japan</u>	<u>06/08/1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/JP00/01608	March 16, 2000	pending

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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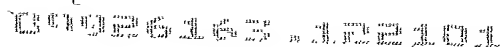
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ICHIKAWA, Chiyo  
MATSUURA, Mitsutaka  
NOGUCHI, Yuji  
SAITO, Yoshimasa  
YAMASHITA, Michio  
TAKATA, Yoko

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35 40 45

Leu Glu His Ala Pro Asp Trp Ala Ile Val Gly Val Gly Leu Thr Gly  
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Ser Asp Arg Ser Lys Lys Lys Ala Glu Glu Phe Lys Ala Gln Asp Cys  
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Leu Tyr Ser Leu Thr Glu Thr Ala Pro Ser Gly Lys Ser Thr Val Arg

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Thr Ile Thr Glu Gly Gly Tyr Asn Ile Asn Glu Thr Thr Gly Ala Phe		
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Pro Ser Thr Val Phe Gly Tyr Val Val Glu Ala Leu Arg Arg Arg Trp		
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Asp Ala Gly Gly Lys Ala Phe Thr Val Met Ser Cys Asp Asn Leu Arg		
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His Asn Gly Asn Val Ala Arg Lys Ala Phe Leu Gly Tyr Ala Lys Ala		
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Arg Asp Pro Glu Leu Ala Lys Trp Ile Glu Glu Asn Ala Thr Phe Pro		
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Asn Gly Met Val Asp Arg Ile Thr Pro Thr Val Ser Ala Glu Ile Ala		
225	230	235
Lys Lys Leu Asn Ala Ala Ser Gly Leu Asp Asp Asp Leu Pro Leu Val		
245	250	255
Ala Glu Asp Phe His Gln Trp Val Leu Glu Asp Gln Phe Ala Asp Gly		
260	265	270
Arg Pro Pro Leu Glu Lys Ala Gly Val Gln Met Val Gly Asp Val Thr		
275	280	285
Asp Trp Glu Tyr Val Lys Ile Arg Met Leu Asn Ala Gly His Val Met		
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